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(54) Title: INCREASING ISOPRENOID BIOSYNTHESIS

(57) Abstract: This invention relates to methods of increasing isoprenoid biosynthesis and/or accumulation, especially in higher plants by genetic manipulation.

INCREASING ISOPRENOID BIOSYNTHESIS

This invention relates to methods of modifying plants and, in particular, to methods of increasing isoprenoid biosynthesis and/or accumulation, especially in higher plants and particularly in crop plants. The invention particularly relates to increasing sterol biosynthesis.

The isoprenoids are a large family (> 10,000 members) of compounds with diverse roles in higher plants. They include the sterols, the plant hormones such as the gibberellins and abscisic acid, various components of photosynthetic pigments, the phytoalexins and a variety of other specialised terpenoids. The isoprenoids are of interest to plant biotechnologists because they contribute to various characteristics such as the nutritional quality, flavour, and colour of crop plants and their products, such as fruits and vegetable oils. For example, the carotenoids lycopene and β-carotene are responsible for the colour of tomatoes and carrots respectively.

Isopentenyl diphosphate (IPP), or "isopentyl pyrophosphate", is the precursor of all isoprenoids in eukaryotes. In animals and yeast, it is derived from acetyl-CoA via a biosynthetic pathway in which mevalonic acid, or mevalonate, is an intermediate. In animal cells, the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid is the overall rate-limiting step for the whole sterol biosynthetic pathway. HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase or HMGR) is the enzyme which catalyses this step and its activity is regulated through phosphorylation by a protein kinase, adenosine 5' phosphate (AMP) - activated protein kinase, AMPK (Clarke, P.R., and Hardie, D. G., (1990), *EMBO J.* **9**, 2439-2446). It is now clear that AMPK is a homologue of the yeast protein kinase SNF1 and of plant SnRK1s (reviewed by Halford, N. G., and Hardie, D. G., (1998) *Plant Molec Biol* **37**, 735-748). HMGR kinase activities have been partially purified from a number of plant species and there is convincing immunological evidence that the major component of these activities corresponds to the SnRK1 protein kinases (Ball, K. L., *et al*, (1995) *FEBS Lett* **37**, 189-192; Barker J. H. A., *et al*, (1996) *Plant Physiology* **112**, 1141-1149).

Phytosterols are plant sterols and can be divided in three groups based on methylation

levels at C4: 4-desmethylsterols or end product sterols, 4α-monomethylsterols and 4, 4-di-methylsterols. The major group is the 4-desmethysterols with β-sitosterol, stigmasterol, and campesterol being the most abundant species. Other 4-desmethylsterols found in oilseeds include brassicasterol and $^{\Delta}$7-avenosterol. Phytosterols can occur in free form (free 3β-hydroxyl group) or as conjugates where the 3-hydroxy group is esterified by fatty acids, phenolic acids such as ferulic acid or with sugar moieties. For the purpose of this description the term sterol refers both to free sterols and conjugated sterols.

Mevalonate synthesis via HMGR is also a key step in isoprenoid biosynthesis in plants, although recent evidence suggests the existence of a second pathway for IPP synthesis (Eisenrach W., *et al*, (1996) *Proc Natl Acad Sci USA* **93**, 6431-6436). In contrast to animal systems, plants contain multiple HMGR genes, the least number found so far being two (HMGR1 and HMGR2) in Arabidopsis (Enjuto M., *et al*, (1994) *Proc Natl Acad Sci USA* **91**, 927-931). Plant HMGR activity is regulated *in vivo* by reversible phosphorylation of the enzyme (Sipat A. B., (1982) *Phytochemistry* **21**, 2613-2618; Russell D. W., *et al*, (1985) *Current topics in Plant Biochemistry* (Randall *et al, Eds)* Columbia MO) as well as transcriptionally (the family members are differentially regulated) (Enjuto M., *et al*., (1994) *supra*). They have divergent N-terminal domains but highly conserved membrane-insertion sequences and C-terminal catalytic domains (Monfar M., *et al*, (1990) *Biochemistry of the mevalonic pathway to terpenoids* (Towers, Stafford, Eds) Plenum Press, NY). The catalytic domain of Arabidopsis HMGR1 has been expressed in an active form in *E.coli* bacteria, and inactivated *in vitro* by the partially purified HMGR kinase activity from cauliflower through phosphorylation of serine-577 (Dale S., *et al*, (1995) *FEBS Lett 361*, 191-195). This phosphorylation site is present in all of the plant HMGRs characterised so far (See Figure 1, Halford N.G., and Hardie D. G.(1998) *supra*).

The accompanying Fig. 1 (from Halford and Hardie *supra*) shows the regulatory phosphorylation sites on HMG-CoA reductases from different plant species. The residues required for recognition by the SnRK1 family are highlighted as follows: (1) phosphorylated serine (P), bold and underlined; (2) hydrophobic residues at P-5, P+4, bold; (3) basic residues at P-4, underlined. All sequences are from the GENBANK/EMBL databases.

An attempt has been made to upregulate HMGR activity in Arabidopsis by introducing additional copies of HMGR1 under the control of a CaMV35S promoter (Re E. B., *et al*, (1995) *Plant J.* 7, 771-784). As well as increasing the copy number of the gene, this also bypasses the transcriptional regulation of its expression. Although the transcript level was increased 40-fold over the level observed in wild-type plants, HMGR activity increased only 3-fold, and no significant change was seen in the accumulation of isoprenoids. In transgenic tobacco plants expressing a HMGR gene from the rubber plant, *Hevea brasiliensis*. (Schaller H., *et al*, (1995) *Plant Phys* **109**, 761-770) and from hamster (Chappell J., *et al*, (1995) *Plant Physiol* **109**, 1337-1343), both using the CaMV35S gene promoter, enzyme activity increased 3-8 fold and in these cases sterol production did increase by 3-6 fold. However, observed changes in enzyme activity and sterol content in plants have so far only been reported in leaf tissue, and not in seed tissue.

Although transformed tobacco plants with the sense construction pGGh-1 shared a significant increase in HMGR activity in the plant extract, it was not possible to discriminate whether the increase was due to chimaeric HMGR or to the endogenous tobacco hMGR as a physiological response of the plant (Godoy-Hernandez G. C *et al* (1998) J. Plant Physiology **53**, 415-424). Also it was not possible to determine whether the alterations in metabolism involved HMGR-related isoprenoid production.

An object of this invention is to increase the levels of isoprenoid, particularly, terpenoid, compounds, and particularly those of nutritional benefit, such as the fat soluble vitamins, like vitamin E and K and sterols, in crop plants and also their products such as rapeseed oil. Both classes of compounds may be efficacious in reducing coronary heart disease. For example, sterols from commonly used edible oils (soybean, rapeseed and sunflower), that is the 4-desmethyl sterols β-sitosterol, stigmasterol, and campesterol, have been shown to have a cholesterol lowering effect (Westrate & Meijer (1998) *Eur J Clin Nutr* **52**: 334-344, Jones *et al.* (1997) *Canadian Journal of Physiology and Pharmacology* **75**, 217-227; Pelletier *et al.* (1995) *Annals and Nutrition and Metabolism* **39**, 291-295) and vitamin E has also been shown to reduce atherosclerotic plaques via oxidation of LDL (Stenvinkel *et al.* (1995) *Kidney International* **5**, 1899-1911; Qiao *et al.* (1993) *Arteriosclerosis and thrombosis* **13**, 1885-1892). Similarly, vitamin K-dependant proteins, are known to play a regulatory role in vascular biology (Pellegrino *et al.* (1996) *Journal of Pediatric*

Gastroenterology and Nutrition **23,** 413-414; Freeman *et al.* (1996) *Journal of Biological Chemistry* **271,** 16227-16236). The proteins are blood coagulation and regulatory proteins that contain γ-carboxyglutamic acid and require calcium for their interaction with cell membranes. γ-carboxyglutamic acid is produced from glutamic acid on the nascent protein chain in a reaction that requires Vitamin K as a cofactor. They include blood clotting factor IX. The data currently suggests that in humans vitamin K-dependent proteins prevent the degeneration of an atherosclerotic vessel wall. In addition, vitamin K is also important in bone metabolism and in the prevention of postmenopausal osteoporosis (Akiyama *et al.* (1999) *Japanese Journal of Pharmacology* **80,** 67-74; Raisz (1999) *Journal of Bone and Mineral Metabolism* **17,** 79-89; Jie *et al.* (1996) *Calcified Tissue International* **59,** 352-356).

The present invention involves introducing novel HMGR genes, in the form of mutant plant and plant/non-plant or different plant chimaeric genes, into plants with the aim of increasing isoprenoid biosynthesis and/or accumulation by uncoupling HMGR from regulation by SnRK1. Transcriptional regulation of the HMGR genes can be avoided by using heterologous promoters. The inventors have shown an increase in seed sterol content, which has not been shown previously.

According to one aspect of the invention there is provided a method of modifying plants, the method comprising modifying a plant HMGR gene which encodes an unmodified HMGR gene product whose activity is regulated so that the modified HMGR gene encodes a modified gene product and placing the modified HMGR gene in a plant or plant cells, in which the modified HMGR gene product is not so regulated.

The unmodified HMGR gene product may be regulated by phosphorylation. Preferably, the modified gene product is no longer subject to regulatory phosphorylation.

The unmodified HMGR gene product may have at least one phosphorylation site. The or each phosphorylation site may include a serine, threonine or tyrosine residue. The or each phosphorylation site may be rendered inactive in the modified HMGR gene product by the replacement of at least one serine, threonine or tyrosine residue of the unmodified gene product with, for example, an alanine residue. Alteration of the phosphorylation site at any

of the positions highlighted in Figure 1 could be as effective as substituting the serine residue, since SnRK1 requires hydrophobic residues at positions +4 and -5 with respect to the serine, and a basic residue at -3 or -4.

The HMGR gene may be further modified to reduce transcriptional regulation. For example, the gene may be modified through the introduction of at least one heterologous promoter. In such a case, the heterologous promoter may, for example, be selected from the CaMV35S and ACP promoters such as, for example, a rapeseed ACP promoter. Homologous promoters can be used but such constructs may be subject to transcriptional regulation.

Alternatively, the plant HMGR gene may be modified by the inclusion of a heterologous sequence for a corresponding HMGR gene from another species

The heterologous sequence may, for example, be derived from yeast. In particular, it may be derived from *S. cerevisae* but other yeasts such as *S. pombe* and *Candida spp.* may be used. Alternatively, it may be derived from another plant species or from fungi or another organism which synthesises isoprenoids.

In either method, the phosphorylation site may fit the consensus sequence:

	-5 -3 ↓ +4
Consensus sequence:	XMXRXXSXXXL
	L K T F
	V H I
	FRX M
	I V

The phosphorylation site may be selected from:

Arabidopsis thaliana 1	HMKYNRSSRDI
Camptotheca acuminata	HMKYNRSNKDV
Catharanthus roseus	HMKYNRSSKDI

Hevea brasiliensis 1	HMKYNRSSKDM
Nicotiana sylvestris	HMKYNRSTKDV
Potato	HMKYNRSIKDI
Rice	HMMYNRSSKDV
Tomato 1	HMKYNRSTKDV

The invention also provides plants and plant reproductive material obtainable by a method of modifying plants according to the invention. The plants may be selected from higher plants such as the crop plants: tobacco, tomatoes, spinach, broccoli, peas, cauliflower and potatoes. Alternatively, the plants may be selected from oil plants such as rapeseed, palm, sunflower, soya bean and tea. The plants may also be selected from monocotyledonous plants, including seeds and the progeny or propagules thereof, for example *Lolium*, *Zea*, *Triticum*, *Sorghum*, *Triticale*, *Bromus*, *Oryzae*, *Avena*, *Hordeum*, *Secale* and *Setaria*, in particular maize, wheat, rice, and barley, as well as dicotyledonous plants, including but not limited to *Fabaceae*, *Brassicaceae*, *Solanum* especially oilseed rape, beans (notably soybeans), sunflower, potatoes, cabbages, spinach, broccoli, peas, cauliflower, tomato, forest trees, roses and tea.

The invention also provides a method of growing plants or plant cells or explants comprising culturing a plant, plant cell, explant or plant reproduction material for example host cultures, obtainable by a method of modifying plants according to the invention. For example, the plant cells and tissue cultures could be made *de novo*, for example by *Agrobacterium tumefaciens*-mediated transformation of plant explants and/or callus culture, or by *Agrobacterium rhizogenes*-mediated transformation of a plant (for example as described by Tepfer D. (1990) Physiologia Plantarum 79, 140146) to produce transgenic hairy roots. Plant cells and tissue cultures can also be produced by generating a transgenic plant, as described, and then inducing callus formation by hormone treatment or hairy root formation by *Agrobacterium rhizogenes* infection.

The invention also provides a method of producing isoprenoids comprising culturing a plant or plant cells or explants and collecting isoprenoids for the plant cells or media; and also isoprenoids obtainable by such a method. Isoprenoids obtainable by such a method

include sterols (such as β-sitosterol, campesterol, stigmasterol, brassicasterol and Δ5-avenosterol), terpenoids (such as fat-soluble vitamins) and carotenoids.

The invention also provides seeds obtainable from plants, plant cells and explants and plant reproduction material according to the invention.

The invention further provides a method of producing isoprenoid-containing oil comprising extracting oil from seeds according to the invention.

The invention also provides a nucleotide sequence encoding a modified HMGR, wherein the amino acid sequence of the modified HMGR is altered relative to the amino acid sequence of unmodified HMGR, by amino acid substitution of at least one serine, threonine or tyrosine residue at a phosphorylation site within the HMGR.

The invention further provides a method for increasing pathogen, fungus and insect and mite pest resistance in plants by increasing the expression of an isoprenoid in the plant by modifying the plant as defined above. Examples of such fungus are *Fusarium*, *Aspergillus*, *Phytopthera*, *Gaeumannomyces*, Downy mildews, *Colletotrichum*, *Cochliobolus*, *Tapesia*, *Magnaporthe*, *Stagonospora*, *Rhynchosporium*, *Septoria*, *Helminthosporium*, and powdery mildews such as *Blumeria* and *Erysiphe*. Examples of insect and mite pests are Homoptera, Diptera, Lepidoptera, Coleoptera, Hemiptera, Hymenoptera, Dictyoptera, Orthoptera, arachnids and mites. The method may also include attracting beneficial species of insects and mites such as any species of Hymenoptera, Odonata, Hemiptera, Coleoptera, Neuroptera, and arachnids including spiders and predatory mite, or their larvae.

The invention also provides a nucleotide sequence encoding a chimaeric HMGR comprising an N-terminal domain-encoding region derived from a plant HMGR nucleotide sequence, and a C-terminal domain-encoding region HMGR nucleotide sequence derived from a different organism, such as a yeast.

The nucleotide sequence may comprise a heterologous promoter such as CaMV355 or ACP.

The invention also provides a HMGR encoded by a nucleotide sequence according to the invention.

The invention further provides a method of increasing the levels of 4-desmethylsterols in plants and seeds by expression of a HMGR according to the invention.

Two particular strategies to produce novel modified HMGR enzymes that are uncoupled from phosphorylation control can be used.

The first strategy involves site directed mutagenesis of the active serine in the enzyme, thereby removing the target for the SnRK1s which phosphorylate the unmodified enzyme.

For example, the sequence of Arabidopsis HMGR1 that encodes Ser^{577} can be altered for example from TCC to GCC, which encodes Ala.

The second strategy involves the construction of a chimaeric gene comprising the catalytic domain-encoding region of an HMGR gene from another isoprenoid-synthesizing organism, for example the yeast HMGR gene, and the N-terminal domain-encoding region of Arabidopsis HMGR1. The gene can then be produced by PCR amplification of the N-terminal region of Arabidopsis HMGR1, which should ensure that the chimaeric HMGR protein is targeted to the correct location in the plant cell and the C-terminal region of yeast HMGR using primers which incorporate a suitable restriction site at the "join", and ligation and cloning of the PCR products. The fusion point is preferably located at the C-terminal end of the linker region between the membrane-spanning domain and the catalytic domain. Other fusions are contemplated and are within the ambit of the skilled worker.
These novel HMGR sequences can be placed in vectors downstream of the constitutive CaMV35S (Odell J. T., *et al*, (1985) *Nature* **313**, 810-812) and acyl carrier protein (ACP) promoters. The ACP promoter De Silva J., *et al*, (1992) *Plant Molec Biol* **18**, 1163-1172 WO92/18634) is active only in seed ("seed specific" - Gallie, D. R., *et al* (1989) *Plant Cell*

1, 301-311; promoters and introduced into tobacco and oilseed rape plants. A suitable ACP promoter-containing vector is pNH12. A suitable CaMV35S promoter containing vector is pJD330. The use of heterologous promotors avoids the transcriptional regulation of the HMGR gene which occurs with the unmodified gene.

The generation of plants and plant tissues in accordance with the invention will now be described, by way of example only, with reference to the following further drawings Figures 2 to 6 in which:

Fig. 2 shows the nucleotide and derived amino acid sequence of *S. cerevisiae* HMG-CoA reductase gene HMGR1 (EMBL database accession number M22002);

Fig. 3 shows the nucleotide and derived amino acid sequence of Arabidopsis HMG-CoA reductase gene HMGR1 (EMBL database accession number J04537);

Fig. 4a shows a schematic diagram of the construction of genes comprising the acyl carrier protein (ACP) gene promoter, nopaline synthase gene terminator (terminator) and either the mutant arabidopis HMGR1 containing the T1799-G (Serine577-alanine) substitution or the chimaeric arabidopsis/yeast HMGR gene.

Fig. 4b shows a schematic diagram of the construction of genes comprising the CaMV35S gene promoter, Ω enhancer sequence (Ω), nopaline synthase gene terminator (terminator) and either the mutant arabidopsis HMGR1 containing the T1799-G (Serine577-alanine) substitution, or the chimaeric arabidopsis/yeast HMGR gene.

Fig. 5A shows a ATHMGRM sequence (i.e. mutant ATHMGR1 sequence) and Fig. 5B shows a chimeric HMGR1 sequence used in constructs of the invention; and

Fig. 6 is an alignment of derived amino acid sequences of HMG-CoA reductases encoded by wild type Arabidopsis gene, HMGR1 (athmgr1), a novel mutant gene, (athmgrm) in which the serine residue (S) at position 577 is replaced with an alanine residue (A), part of the yeast (Saccharomyces cerevisiae) wild type gene (schmgr1) and a novel chimaeric gene comprising the N-terminal membrane-spanning part of the Arabidopsis HMGR1 gene and the C-terminal catalytic part of the yeast HMGR1 gene. Matching residues are highlighted with a black background. Residue numbers are given on the right.

Fig. 7 is an alignment of derived amino acid sequences of HMG-CoA reductases encoded by wild type Arabidopsis gene, HMGR1 (athmgr1) and the mutant gene, (athmgrm) in which the serine residue (S) at position 577 is replaced with an alanine residue (A). Matching residues are highlighted with a black background.

Fig, 8 is an alignment of derived amino acid sequences of HMG-CoA reductases encoded by wild type Arabidopsis gene, HMGR1 (athmgr1), yeast (Saccharomyces cerevisiae) wild type gene (schmgr1) and a novel chimaeric gene comprising the N-terminal membrane-spanning part of the Arabidopsis HMGR1 gene and the C-terminal catalytic part of the yeast HMGR1 gene. For clarity, only the N-terminal portion of the Arabidopsis protein and C-terminal portion of the yeast protein are included, but all of the novel chimaeric protein is shown. Matching residues are highlighted with a black background. Residue numbers are given on the right.

Fig. 9 is a bar chart comparing levels of EJD25 and MAS1 sterols in leaf material.

Fig. 10 is a bar chart comparing levels EJD25, ENH7 and MAS1 sterols in seed material.

1. Generation of engineered plants

Modified plants can be produced according to two strategies: either the HMGR gene is modified by site directed mutagenesis so as to encode a modified gene product which is resistant to phosphorylation because it lacks a specific serine residue (serine 577) or a more substantial modification is made to the HMGR gene so that a C terminal portion in the HMGR gene product is replaced by a coding region from a different organism.

a. Site directed mutagenesis of HMGR gene

Primers ATACAATAGAGCCAGCCGAGAC and GTCTCGGCTGGCTCTATTGTAT are generated and used for site-directed mutagenesis (for example using Stratagene Quickchange system) of the ATHMGR1 sequence to replace T^{1799} with G, causing a $Serine^{577}$ to alanine substitution in the encoded protein (See Fig. 5A). Alterations may be also performed to cause substitutions at positions 572, 573, 574 or 581 of the amino acid sequence, corresponding to the positions highlighted in Figure. 1.

The modified gene, ATHMGRM, is shown schematically in Fig. 4 and its sequence is shown in Fig. 5. A. The modified HMGR1 coding sequence is under the control of the ACP promoter. The terminator is the nopaline synthase (nos) termination sequence. The modified HMGR gene is then introduced into plants using conventional *Agrobacterium tumefaciens* - mediated transformation (Bevan, M, 1984 *infra*).

b. Production of chimaeric HMGR genes

ATHMGR1 sequences can be amplified from Arabidopsis total RNA by rtPCR.

SCHMGR1 sequences are amplified from yeast total RNA by rtPCR. The following oligonucleotide primers can be used:

1. ACGTCCATGGATCTCCGTCGGAGGC

2. ACGTGAATTCAGATTCAGATCATGT

This pair are used to amplify the full-length ATHMGR1 sequence (71 – 1858 in the sequence of Figure 3 below) and to incorporate *Nco*I and *Eco*RI restriction sites

3. AAACCTGCAGAGAACAAAGAGGTCGCC

4. ACGTGAATTCGACGTATGACTAAGTTTAGG

are used to amplify the catalytic domain of SCHMGR1 (encoded by base pairs 1970 - 3298 in sequence below) and to incorporate *Pst*I and *Eco*RI restriction sites within the amplified DNA.

5. GTCTTCTGCAGGAAGCGATTCGGT

This oligonucleotide together with oligonucleotide ACGTCCATGGATCTCCGTCGGAGGC can be used to amplify the targeting domain of AtHMGR1 (71 – 578 below)

Two additional constructs can be made by cloning the mutant and chimaeric HMGR sequences downstream of an Ω-enhanced CaMV35S promoter in plasmid PJD330 (Gallie, D. R., *et al* (1989) *supra*). This promoter drives expression in a constitutive manner. This

involves amplification of the sequences with the original 5' oligonucleotides and the following 3' oligonucleotides:

Mutant arabidopsis HMGR:

ACGTCCCGGGAGATTCAGATCATGT

Chimaeric HMGR:

ACGTCCCGGGACGTATGACTAAGTTTAGGA

These will introduce a SmaI site at the 3' end (see Fig. 4b).

The resulting constructs are then introduced into plants as described above.

Mutant and chimaeric HMGR genes have been produced. The mutant gene was produced by converting T^{1799} (Fig. 3) of the *Arabidopsis* gene to a G, by site directed mutagenesis, leading to a Serine to Alanine substitution in the SnRK phosphorylation site. This should remove the encoded protein from phosphorylational control by the kinase. The chimaeric gene was prepared by joining the targeting domain of the Arabidopsis gene to the catalytic domain of the yeast gene and lacks the SNF1/SnRK1 phosphorylation site. The HMGR protein is not under the control of the Snf1 kinase in yeast. The entire nucleotide sequences of these genes have been checked against those published. The sequences have been placed downstream of cauliflower mosaic virus 35S RNA and ACP promoters by cloning them into JD330 and NH12 plasmids respectively, to make a total of 4 chimaeric gene constructs, 35S-mutant HMGR (MAS), 35S-chimaeric HMGR (ASS), ACP-mutant HMGR (MAE) and ACP-chimaeric HMGR (MASE).

c. Plasmids containing the chimaeric gene constructs have been used to transform tobacco (SR1) using Agrobacterium-mediated transformation. Control plants have been produced containing promoter and terminator sequences without the HMGR inserts. The numbers of transgenic plants generated is given in Table 1:

Table 1

Construct	Number of Plant
35S-mutant HMGR	33

35S-chimaeric HMGR	24
35S-empty cassette	21
ACP-mutant HMGR	44
ACP-chimaeric HMGR	18
ACP-empty cassetts	11

The transgenic plants have been analysed by RT-PCR, measurement of HMGR activity, and also analysis of sterol content. 16 plants have been analysed for sterol content and at least one, MAS1, containing the 35S mutant HMGR construct has been found to have a higher sterol content than controls in both leaves and seeds. Analyses of its sterol content compared with EJD25 (35S - empty cassettes) and ENH7 (ACP-empty cassettes) are shown in Figs. 9 and 10.

The inventors have shown that transforming a plant with an HMGR gene encoding a protein lacking in the target phosphorylation site recognized by the SnRK1 protein kinase results in increased sterol biosynthesis.

d. Production of other plants which have been engineered

Other plants such as oilseed rape can be produced by transformation using *Agrobacterium tumefaciens* (See Bevan M. *Nucl. Acids Res.* 1984; **12,** 8711-8721; Horsch. R. B., (1985) *Science* **227,** 1229-1231).

2. Generation of plant cells

a. Production of engineered tobacco cells

Plant cells can be produced from the plants described in Example 1 above or they could be made *de novo*, for example by *Agrobacterium tumefaciens*-mediated transformation of a callus culture, or by *Agrobacterium rhizogenes*-mediated transformation of a plant (for example as described in Tepfer, D. (1990) Physiologia Plantarum 79: 140-146) to produce hairy roots. Alternatively, plant and tissue cultures can be generated by producing a transgenic plant, as described, then inducing callus formation by hormone treatment or hairy root formation by *Agrobacterium rhizogenes* infection. Calluses can be produced as described for example by Mar *et al* (1997) Plant Molecular Biology **34**, 31-43 with the technique being adapted by the skilled worker. Generally, calluses can be induced from

many different plant tissues by treatment with an auxin (usually 2,4-dichlorophenoxyacetic acid).

The production of isoprenoids in hairy root cultures is well known. Examples of isoprenoid production in hairy root cultures are given by: Sim, S. J. *et al* (1994), *Journal of Fermentation and Bioengineering* **78**, 229-234; Takeda, T. *et al* (1994) *Chem. Pharm. Bull.* **42**, 730-732; Delbecque *et al* (1995) *Eur. J. Entomol.* **92**, 301-307; Hu, Z-B. and Alferman, A. W. (1993) *Phytochemistry* **32**, 699-703; and Sato K. *et al* (1991) *Phytochemistry* **30**, 1507-1510 by way of example.

b. Culture of plant cells

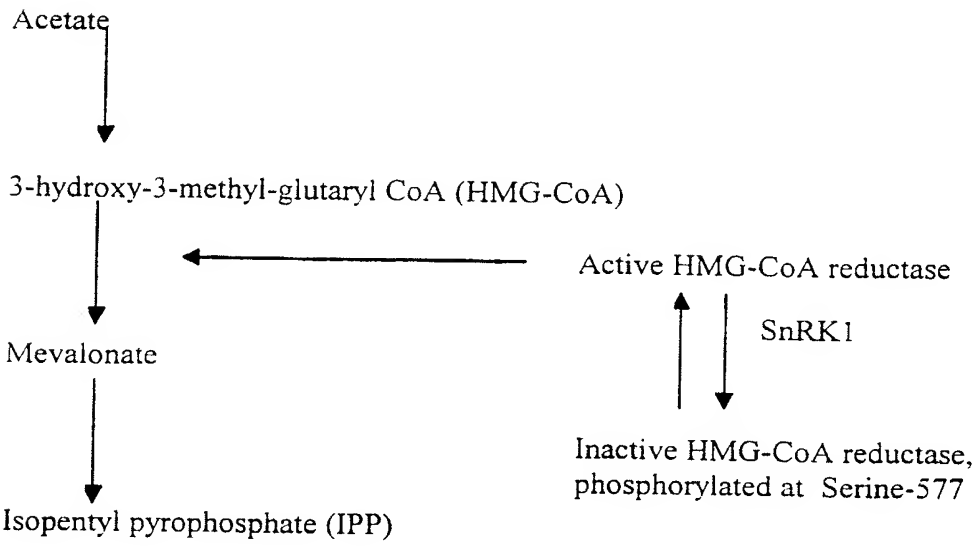
Plant cells produced as described above can be cultured under normal conditions.

3. Production of isoprenoids.

Plants and plant cells and explants produced as described above were grown under normal conditions and HMGR activity measured by the radiochemical method described by Chappell *et al.* (1995) (*Plant Physiology* **109**, 1337-1343). Sterol content was analysed by gas chromatography-mass spectroscopy (gc-ms)

A schematic representation of the preparation of isopentyl pyrophosphate is shown in the following flow diagram:

Flow diagram



IPP is the precursor of all isoprenoids, including sterols, gibberellins, ABA, phytoalexins, various pigments, carotenoids, fat-soluble vitamins (e.g E and K) and more.

Whilst the invention has been described in relation to increasing isoprenoids in oilseeds to enhance the value of the oil, the invention can be used to improve food quality or nutritional value of many crops. Other applications include increasing pathogen and insect resistance of plants, and in the production of pharmaceuticals, fragrances and other non-food substances in plants and plant callus or cell or explant cultures.

Claims

1. A method of modifying plants, the method comprising modifying a plant HMGR gene which encodes an unmodified HMGR gene product whose activity is regulated so that the modified HMGR gene encodes a modified HMGR gene product, and placing the modified HMGR gene in a plant or plant cells, in which the modified HMGR gene product is not so regulated.

2. A method according to claim 1 in which the unmodified HMGR gene product is regulated by phosphorylation.

3. A method according to claim 1 or 2 in which the modified HMGR gene product is no longer subject to regulatory phosphorylation.

4. A method according to claim 1, 2 or 3 in which the unmodified HMGR gene product has at least one phosphorylation site.

5. A method according to claim 4 in which the or each phosphorylation site includes a serine, threonine or tyrosine residue.

6. A method according to claim 5 in which the phosphorylation site has been rendered inactive in the modified HMGR gene product by the replacement of at least one serine threonine or tyrosine residue of the unmodified gene product.

7. A method according to claim 6 in which at least one serine, threonine or tyrosine residue is replaced by at least one alanine or other residue.

8. A method according to any preceding claim in which the HMGR gene is modified to reduce transcriptional regulation.

9. A method according to claim 8 in which the HMGR gene is modified through the introduction of at least one heterologous promoter.

10. A method according to claim 9 in which the heterologous promoter is selected from the CaMV35S and ACP promoters.

11. A method according to claim 9 wherein the ACP promoter is a rapeseed ACP promoter.

12. A method according to claim 1 in which the method comprises modifying a plant HMGR gene which encodes an unmodified HMGR gene product whose activity is regulated, by the inclusion of a heterologous sequence for a corresponding HMGR gene from another species.

13. A method according to claim 12 in which the heterologous sequence is derived from another plant species, yeast or fungi or another isoprenoid-producing organism.

14. A method according to claim 12 or 13 and in which the heterologous sequence is derived from a yeast, in which the yeast is selected from *S. cerevisae*, *S. pombe* and Candida spp.

15. A method according to any one of claims 4 to 7 in which the phosphorylation site fits the consensus sequence:

	-5 -3 ↓ +4
Consensus sequence:	XMXRXX**S**XXXL
	L K **T** F
	V H I
	FRX M
	I V

16. A method according to claim 15 wherein the phosphorylation site is selected from:

Arabidopsis thaliana 1	HM<u>K</u>YNR<u>S</u>SRD**I**
Camptotheca acuminata	HM<u>K</u>YNR<u>S</u>NKD**V**
Catharanthus roseus	HM<u>K</u>YNR<u>S</u>SKD**I**

Hevea brasiliensis 1	HMKYNRSSKDM
Nicotiana sylvestris	HMKYNRSTKDV
Potato	HMKYNRSIKDI
Rice	HMMYNRSSKDV
Tomato 1	HMKYNRSTKDV

17. A method according to any one of claims 1 to 16 in which the plant is selected from higher plants.

18. A method according to claim 17 in which the higher plant is a crop plant.

19. A method according to claim 18 in which the crop plant is selected from tobacco, tomatoes, vegetables such as spinach, broccoli, peas cauliflower and potatoes, oil plants such as rapeseed, palm, sunflower, soybean, tea, maize, wheat, rice, barley, beans, sunflower, cabbage, tomato, forest trees and roses.

20. Plants, plant cells and explants and plant reproductive material obtainable by a method according to any preceding claim.

21. A method of growing plants or plant cells or explants comprising culturing a plant, plant cell, explant or plant reproduction material according to claim 20.

22. A method of producing isoprenoids comprising culturing a plant, plant cell or explant according to claim 20 and collecting isoprenoids from the plant, plant cell or explant or media.

23. A method according to claim 22 in which the plant is selected from higher plants.

24. A method according to claim 23 in which the higher plant is a crop plant.

25. A method according to claim 24 in which the crop plant is selected from tobacco, tomatoes, vegetables such as spinach, broccoli, peas, cauliflower and potatoes, oil plants such as rapeseed, palm, sunflower, soybean, tea, maize, wheat, rice, barley, beans, sunflower, cabbage, forest trees and roses.

26. A method according to any one of claims 22 to 25 in which the isoprenoid is a sterol, a terpenoid or a carotenoid.

27. A method according to any one of claims 22 to 26 in which the isoprenoid is a fat soluble vitamin.

28. A method according to claim 26 and in which the isoprenoid is a sterol in which the sterol is β-sitosterol, campesterol, stigmasterol, brassicasterol or Δ5-avenosterol.

29. Isoprenoids obtainable by a method according to any one of claims 22 to 25.

30. Seeds obtained from plants, plant cells and explants and plant reproductive material according to claim 20.

31. A method of producing isoprenoid-containing oil comprising extracting the oil from seeds according to claim 30.

32. A nucleotide sequence encoding a modified HMGR, wherein the amino acid sequence of the modified HMGR is altered relative to the amino acid sequence of unmodified HMGR by amino acid substitution of at least one serine, threonine or tyrosine residue at a phosphorylation site within the HMGR.

33. A nucleotide sequence encoding a chimaeric HMGR comprising an N-terminal domain-encoding region derived from a plant HMGR nucleotide sequence, and a C-terminal domain-encoding region HMGR nucleotide sequence derived from a different organism.

34. A nucleotide sequence according to claim 33 wherein the different organism is a yeast.

35. A nucleotide sequence according to any one of claims 32 to 34 comprising a heterologous promoter.

36. A nucleotide sequence according to claim 35 wherein the heterologous promoter is CaMV355 or ACP.

37. A HMGR encoded by a nucleotide sequence according to any one of claims 32 to 36.

38. A method of increasing the levels of 4-desmethylsterols in plants and seeds by expression of a HMGR according to claim 37.

39. A method of increasing pathogen resistance in a plant, the method comprising increasing expression of an isoprenoid in the plant by a method of modifying the plant according to any one of claims 1 to 19.

40. A method according to claim 39 in which the plant is selected from monocotyledonous plants, including seeds and the progeny or propagules thereof, for example *Lolium*, *Zea*, *Triticum*, *Sorghum*, *Triticale*, *Bromus*, *Oryzae*, *Avena*, *Hordeum*, *Secale* and *Setaria*, in particular maize, wheat, rice, and barley, as well as dicotyledonous plants, including but not limited to *Fabaceae*, *Brassicaceae*, Solanum especially oilseed rape, beans (notably soybeans), sunflower, potatoes, cabbages, spinach, broccoli, peas, cauliflower, tomato, forest trees, roses and tea.

41. A method according to claim 39 in which the pathogen is a fungus.

42. A method according to claim 41 in which the fungus is any species of *Fusarium*, *Aspergillus*, *Phytopthera*, *Gaeumannomyces*, Downy mildews, *Colletotrichum*, *Cochliobolus*, *Tapesia*, *Magnaporthe*, *Stagonospora*, *Rhynchosporium*, *Septoria*, *Helminthosporium*, and powdery mildews such as *Blumeria* and *Erysiphe*.

43. A method of increasing resistance to insect and mite pests in a plant, the method comprising increasing expression of an isoprenoid in the plant by a method of modifying the plant according to any one of claims 1 to 19.

44. A method according to claim 43 in which the insect is any species of Homoptera, Diptera, Lepidoptera, Coleoptera, Hemiptera, Hymenoptera, Dictyoptera, Orthoptera, arachnids and mites.

45. A method according to claim 43 in which a beneficial insect or a mite, such as any species of Hymenoptera, Odonata, Hemiptera, Coleoptera, Neuroptera, and arachnids including spiders and predatory mite, or their larvae is attracted to the plant.

Arabidopsis thaliana 1	HMKYNRSSRDI
Camptotheca acuminata	HMKYNRSNKDV
Catharanthus roseus	HMKYNRSSKDI
Hevea brasiliensis 1	HMKYNRSSKDM
Nicotiana sylvestris	HMKYNRSTKDV
Potato	HMKYNRSIKDI
Rice	HMMYNRSSKDV
Tomato 1	HMKYNRSTKDV

FIG. 1

FIG. 2

Yeast HMGR1

```
   1  TTTATTAACTTATTTTTTTCTTCTTTCTACCCAATTCTAGTCAGGAAAAGACTAAGGGCT   60

  61  GGAACATAGTGTATCATTGTCTAATTGTTGATACAAAGTAGATAAATACATAAAACAAGC   120

      M  P  P  L  F  K  G  L  K  Q  M  A  K  P  I  A  Y  V  S  R
 121  ATGCCGCCGCTATTCAAGGGACTGAAACAGATGGCAAAGCCAATTGCCTATGTTTCAAGA   180

      F  S  A  K  R  P  I  H  I  I  L  F  S  L  I  I  S  A  F  A
 181  TTTTCGGCGAAACGACCAATTCATATAATACTTTTTTCTCTAATCATATCCGCATTCGCT   240

      Y  L  S  V  I  Q  Y  Y  F  N  G  W  Q  L  D  S  N  S  V  F
 241  TATCTATCCGTCATTCAGTATTACTTCAATGGTTGGCAACTAGATTCAAATAGTGTTTTT   300

      E  T  A  P  N  K  D  S  N  T  L  F  Q  E  C  S  H  Y  Y  R
 301  GAAACTGCTCCAAATAAAGACTCCAACACTCTATTTCAAGAATGTTCCCATTACTACAGA   360

      D  S  S  L  D  G  W  V  S  I  T  A  H  E  A  S  E  L  P  A
 361  GATTCCTCTCTAGATGGTTGGGTATCAATCACCGCGCATGAAGCTAGTGAGTTACCAGCC   420

      P  H  H  Y  Y  L  L  N  L  N  F  N  S  P  N  E  T  D  S  I
 421  CCACACCATTACTATCTATTAAACCTGAACTTCAATAGTCCTAATGAAACTGACTCCATT   480

      P  E  L  A  N  T  V  F  E  K  D  N  T  K  Y  I  L  Q  E  D
 481  CCAGAACTAGCTAACACGGTTTTTGAGAAAGATAATACAAAATATATTCTGCAAGAAGAT   540

      L  S  V  S  K  E  I  S  S  T  D  G  T  K  W  R  L  R  S  D
 541  CTCAGTGTTTCCAAAGAAATTTCTTCTACTGATGGAACGAAATGGAGGTTAAGAAGTGAC   600

      R  K  S  L  F  D  V  K  T  L  A  Y  S  L  Y  D  V  F  S  E
 601  AGAAAAAGTCTTTTCGACGTAAAGACGTTAGCATATTCTCTCTACGATGTATTTTCAGAA   660

      N  V  T  Q  A  D  P  F  D  V  L  I  M  V  T  A  Y  L  M  M
 661  AATGTAACCCAAGCAGACCCGTTTGACGTCCTTATTATGGTTACTGCCTACCTAATGATG   720

      F  Y  T  I  F  G  L  F  N  D  M  R  K  T  G  S  N  F  W  L
 721  TTCTACACCATATTCGGCCTCTTCAATGACATGAGGAAGACCGGGTCAAATTTTTGGTTG   780

      S  A  S  T  V  V  N  S  A  S  S  L  F  L  A  L  Y  V  T  Q
 781  AGCGCCTCTACAGTGGTCAATTCTGCATCATCACTTTTCTTAGCATTGTATGTCACCCAA   840

      C  I  L  G  K  E  V  S  A  L  T  L  F  E  G  L  P  F  I  V
 841  TGTATTCTAGGCAAAGAAGTTTCCGCATTAACTCTTTTTGAAGGTTTGCCTTTCATTGTA   900

      V  V  V  G  F  K  H  K  I  K  I  A  Q  Y  A  L  E  K  F  E
 901  GTTGTTGTTGGTTTCAAGCACAAAATCAAGATTGCCCAGTATGCCCTGGAGAAATTTGAA   960

      R  V  G  L  S  K  R  I  T  T  D  E  I  V  F  E  S  V  S  E
 961  AGAGTCGGTTTATCTAAAAGGATTACTACCGATGAAATCGTTTTTGAATCCGTGAGCGAA   1020

      E  G  G  R  L  I  Q  D  H  L  L  C  I  F  A  F  I  G  C  S
1021  GAGGGTGGTCGTTTGATTCAAGACCATTTGCTTTGTATTTTTGCCTTTATCGGATGCTCT   1080

      M  Y  A  H  Q  L  K  T  L  T  N  F  C  I  L  S  A  F  I  L
1081  ATGTATGCTCACCAATTGAAGACTTTGACAAACTTCTGCATATTATCAGCATTTATCCTA   1140
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```
      I  F  E  L  I  L  T  P  T  F  Y  S  A  I  L  A  L  R  L  E
1141  ATTTTTGAATTGATTTTAACTCCTACATTTTATTCTGCTATCTTAGCGCTTAGACTGGAA  1200

      M  N  V  I  H  R  S  T  I  I  K  Q  T  L  E  E  D  G  V  V
1201  ATGAATGTTATCCACAGATCTACTATTATCAAGCAAACATTAGAAGAAGACGGTGTTGTT  1260

      P  S  T  A  R  I  I  S  K  A  E  K  K  S  V  S  S  F  L  N
1261  CCATCTACAGCAAGAATCATTTCTAAAGCAGAAAAGAAATCCGTATCTTCTTTCTTAAAT  1320

      L  S  V  V  V  I  I  M  K  L  S  V  I  L  L  F  V  F  I  N
1321  CTCAGTGTGGTTGTCATTATCATGAAACTCTCTGTCATACTGTTGTTTGTCTTCATCAAC  1380

      F  Y  N  F  G  A  N  W  V  N  D  A  F  N  S  L  Y  F  D  K
1381  TTTTATAACTTTGGTGCAAATTGGGTCAATGATGCCTTCAATTCATTGTACTTCGATAAG  1440

      E  R  V  S  L  P  D  F  I  T  S  N  A  S  E  N  F  K  E  Q
1441  GAACGTGTTTCTCTACCAGATTTTATTACCTCGAATGCCTCTGAAAACTTTAAAGAGCAA  1500

      A  I  V  S  V  T  P  L  L  Y  Y  K  P  I  K  S  Y  Q  R  I
1501  GCTATTGTTAGTGTCACCCCATTATTATATTACAAACCCATTAAGTCCTACCAACGCATT  1560

      E  D  M  V  L  L  L  L  R  N  V  S  V  A  I  R  D  R  F  V
1561  GAGGATATGGTTCTTCTATTGCTTCGTAATGTCAGTGTTGCCATTCGTGATAGGTTCGTC  1620

      S  K  L  V  L  S  A  L  V  C  S  A  V  I  N  V  Y  L  L  N
1621  AGTAAATTAGTTCTTTCCGCCTTAGTATGCAGTGCTGTCATCAATGTGTATTTATTGAAT  1680

      A  A  R  I  H  T  S  Y  T  A  D  Q  L  V  K  T  E  V  T  K
1681  GCTGCTAGAATTCATACCAGTTATACTGCAGACCAATTGGTGAAAACTGAAGTCACCAAG  1740

      K  S  F  T  A  P  V  Q  K  A  S  T  P  V  L  T  N  K  T  V
1741  AAGTCTTTTACTGCTCCTGTACAAAAGGCTTCTACACCAGTTTTAACCAATAAAACAGTC  1800

      I  S  G  S  K  V  K  S  L  S  S  A  Q  S  S  S  S  G  P  S
1801  ATTTCTGGATCGAAAGTCAAAAGTTTATCATCTGCGCAATCGAGCTCATCAGGACCTTCA  1860

      S  S  S  E  E  D  D  S  R  D  I  E  S  L  D  K  K  I  R  P
1861  TCATCTAGTGAGGAAGATGATTCCCGCGATATTGAAAGCTTGGATAAGAAAATACGTCCT  1920

      L  E  E  L  E  A  L  L  S  S  G  N  T  K  Q  L  K  N  K  E
1921  TTAGAAGAATTAGAAGCATTATTAAGTAGTGGAAATACAAAACAATTGAAGAACAAAGAG  1980

      V  A  A  L  V  I  H  G  K  L  P  L  Y  A  L  E  K  K  L  G
1981  GTCGCTGCCTTGGTTATTCACGGTAAGTTACCTTTGTACGCTTTGGAGAAAAAATTAGGT  2040

      D  T  T  R  A  V  A  V  R  R  K  A  L  S  I  L  A  E  A  P
2041  GATACTACGAGAGCGGTTGCGGTACGTAGGAAGGCTCTTTCAATTTTGGCAGAAGCTCCT  2100

      V  L  A  S  D  R  L  P  Y  K  N  Y  D  Y  D  R  V  F  G  A
2101  GTATTAGCATCTGATCGTTTACCATATAAAAATTATGACTACGACCGCGTATTTGGCGCT  2160

      C  C  E  N  V  I  G  Y  M  P  L  P  V  G  V  I  G  P  L  V
2161  TGTTGTGAAAATGTTATAGGTTACATGCCTTTGCCCGTTGGTGTTATAGGCCCCTTGGTT  2220

      I  D  G  T  S  Y  H  I  P  M  A  T  T  E  G  C  L  V  A  S
2221  ATCGATGGTACATCTTATCATATACCAATGGCAACTACAGAGGGTTGTTTGGTAGCTTCT  2280

      A  M  R  G  C  K  A  I  N  A  G  G  G  A  T  T  V  L  T  K
2281  GCCATGCGTGGCTGTAAGGCAATCAATGCTGGCGGTGGTGCAACAACTGTTTTAACTAAG  2340
```

FIG. 2CONT'D

```
      D  G  M  T  R  G  P  V  V  R  F  P  T  L  K  R  S  G  A  C
2341  GATGGTATGACAAGAGGCCCAGTAGTCCGTTTCCCAACTTTGAAAAGATCTGGTGCCTGT  2400

      K  I  W  L  D  S  E  E  G  Q  N  A  I  K  K  A  F  N  S  T
2401  AAGATATGGTTAGACTCAGAAGAGGGACAAAACGCAATTAAAAAAGCTTTTAACTCTACA  2460

      S  R  F  A  R  L  Q  H  I  Q  T  C  L  A  G  D  L  L  F  M
2461  TCAAGATTTGCACGTCTGCAACATATTCAAACTTGTCTAGCAGGAGATTTACTCTTCATG  2520

      R  F  R  T  T  T  G  D  A  M  G  M  N  M  I  S  K  G  V  E
2521  AGATTTAGAACAACTACTGGTGACGCAATGGGTATGAATATGATTTCTAAAGGTGTCGAA  2580

      Y  S  L  K  Q  M  V  E  E  Y  G  W  E  D  M  E  V  V  S  V
2581  TACTCATTAAAGCAAATGGTAGAAGAGTATGGCTGGGAAGATATGGAGGTTGTCTCCGTT  2640

      S  G  N  Y  C  T  D  K  K  P  A  A  I  N  W  I  E  G  R  G
2641  TCTGGTAACTACTGTACCGACAAAAAACCAGCTGCCATCAACTGGATCGAAGGTCGTGGT  2700

      K  S  V  V  A  E  A  T  I  P  G  D  V  V  R  K  V  L  K  S
2701  AAGAGTGTCGTCGCAGAAGCTACTATTCCTGGTGATGTTGTCAGAAAAGTGTTAAAAAGT  2760

      D  V  S  A  L  V  E  L  N  I  A  K  N  L  V  G  S  A  M  A
2761  GATGTTTCCGCATTGGTTGAGTTGAACATTGCTAAGAATTTGGTTGGATCTGCAATGGCT  2820

      G  S  V  G  G  F  N  A  H  A  A  N  L  V  T  A  V  F  L  A
2821  GGGTCTGTTGGTGGATTTAACGCACATGCAGCTAATTTAGTGACAGCTGTTTTCTTGGCA  2880

      L  G  Q  D  P  A  Q  N  V  E  S  S  N  C  I  T  L  M  K  E
2881  TTAGGACAAGATCCTGCACAAAATGTTGAAAGTTCCAACTGTATAACATTGATGAAAGAA  2940

      V  D  G  D  L  R  I  S  V  S  M  P  S  I  E  V  G  T  I  G
2941  GTGGACGGTGATTTGAGAATTTCCGTATCCATGCCATCCATCGAAGTAGGTACCATCGGT  3000

      G  G  T  V  L  E  P  Q  G  A  M  L  D  L  L  G  V  R  G  P
3001  GGTGGTACTGTTCTAGAACCACAAGGTGCCATGTTGGACTTATTAGGTGTAAGAGGCCCG  3060

      H  A  T  A  P  G  T  N  A  R  Q  L  A  R  I  V  A  C  A  V
3061  CATGCTACCGCTCCTGGTACCAACGCACGTCAATTAGCAAGAATAGTTGCCTGTGCCGTC  3120

      L  A  G  E  L  S  L  C  A  A  L  A  A  G  H  L  V  Q  S  H
3121  TTGGCAGGTGAATTATCCTTATGTGCTGCCCTAGCAGCCGGCCATTTGGTTCAAAGTCAT  3180

      M  T  H  N  R  K  P  A  E  P  T  K  P  N  N  L  D  A  T  D
3181  ATGACCCACAACAGGAAACCTGCTGAACCAACAAAACCTAACAATTTGGACGCCACTGAT  3240

      I  N  R  L  K  D  G  S  V  T  C  I  K  S  *
3241  ATAAATCGTTTGAAAGATGGGTCCGTCACCTGCATTAAATCCTAAACTTAGTCATACGTC  3300

3301  ATTGGTATTCTCTTGAAAAAGAAGCACAACAGCACCATGTGTTACGTAAAATATTTACTT  3360
```

FIG. 2CONT'D

FIG. 3

Arabidopsis HMGR1

```
   1 ATCACGCCACCTCACCACCTCTCTCCTCTCTCCTCTCTCTCCCCCCTGGAGAGATTATTC   60

                 M  D  L  R  R  R  P  P  K  P  P  V  T  N  N  N  N
  61 ATTCCCTCCAATGGATCTCCGTCGGAGGCCTCCTAAACCACCGGTTACCAACAACAACAA  120

              S  N  G  S  F  R  S  Y  Q  P  R  T  S  D  D  D  H  R  R  R
 121 CTCCAACGGATCTTTCCGTTCTTATCAGCCTCGCACTTCCGATGACGATCATCGTCGCCG  180

           A  T  T  I  A  P  P  P  K  A  S  D  A  L  P  L  P  L  Y  L
 181 GGCTACAACAATTGCTCCTCCACCGAAAGCATCCGACGCGCTTCCTCTTCCGTTATATCT  240

           T  N  A  V  F  F  T  L  F  F  S  V  A  Y  Y  L  L  H  R  W
 241 CACAAACGCCGTTTTCTTCACGCTCTTCTTCTCCGTCGCGTATTACCTCCTCCACCGGTG  300

           R  D  K  I  R  Y  N  T  P  L  H  V  V  T  I  T  E  L  G  A
 301 GCGTGACAAGATCCGTTACAATACGCCTCTTCACGTCGTCACTATCACAGAACTCGGCGC  360

           I  I  A  L  I  A  S  F  I  Y  L  L  G  F  F  G  I  D  F  V
 361 CATTATTGCTCTCATCGCTTCGTTTATCTATCTCCTAGGGTTTTTTGGTATTGACTTTGT  420

           Q  S  F  I  S  R  A  S  G  D  A  W  D  L  A  D  T  I  D  D
 421 TCAGTCATTTATCTCACGTGCCTCTGGTGATGCTTGGGATCTCGCCGATACGATCGATGA  480

           D  D  H  R  L  V  T  C  S  P  P  T  P  I  V  S  V  A  K  L
 481 TGATGACCACCGCCTTGTCACGTGCTCTCCACCGACTCCGATCGTTTCCGTTGCTAAATT  540

           P  N  P  E  P  I  V  T  E  S  L  P  E  E  D  E  E  I  V  K
 541 ACCTAATCCGGAACCTATTGTTACCGAATCGCTTCCTGAGGAAGACGAGGAGATTGTGAA  600

           S  V  I  D  G  V  I  P  S  Y  S  L  E  S  R  L  G  D  C  K
 601 ATCGGTTATCGACGGAGTTATTCCATCGTACTCGCTTGAATCTCGTCTCGGTGATTGCAA  660

           R  A  A  S  I  R  R  E  A  L  Q  R  V  T  G  R  S  I  E  G
 661 AAGAGCGGCGTCGATTCGTCGTGAGGCGTTGCAGAGAGTCACCGGGAGATCGATTGAAGG  720

           L  P  L  D  G  F  D  Y  E  S  I  L  G  Q  C  C  E  M  P  V
 721 GTTACCGTTGGATGGATTTGATTATGAATCGATTTTGGGGCAATGCTGTGAGATGCCTGT  780

              G  Y  I  Q  I  P  V  G  I  A  G  P  L  L  L  D  G  Y  E  Y
 781 TGGATACATTCAGATTCCTGTTGGGATTGCTGGTCCATTGTTGCTTGATGGTTATGAGTA  840

           S  V  P  M  A  T  T  E  G  C  L  V  A  S  T  N  R  G  C  K
 841 CTCTGTTCCTATGGCTACAACCGAAGGTTGTTTGGTTGCTAGCACTAACAGAGGCTGCAA  900

           A  M  F  I  S  G  G  A  T  S  T  V  L  K  D  G  M  T  R  A
 901 GGCTATGTTTATCTCTGGTGGCGCCACCAGTACCGTTCTTAAGGACGGTATGACCCGAGC  960

           P  V  V  R  F  A  S  A  R  R  A  S  E  L  K  F  F  L  E  N
 961 ACCTGTTGTTCGGTTCGCTTCGGCGAGACGAGCTTCGGAGCTTAAGTTTTTCTTGGAGAA 1020

           P  E  N  F  D  T  L  A  V  V  F  N  R  S  S  R  F  A  R  L
1021 TCCAGAGAACTTTGATACTTTGGCAGTAGTCTTCAACAGGTCGAGTAGATTTGCAAGACT 1080
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                Q  S  V  K  C  T  I  A  G  K  N  A  Y  V  R  F  C  C  S  T
1081  GCAAAGTGTTAAATGCACAATCGCGGGGAAGAATGCTTATGTAAGGTTCTGTTGTAGTAC  1140

                G  D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  L  E  Y  L
1141  TGGTGATGCTATGGGGATGAATATGGTTTCTAAAGGTGTGCAGAATGTTCTTGAGTATCT  1200

                T  D  D  F  P  D  M  D  V  I  G  I  S  G  N  F  C  S  D  K
1201  TACCGATGATTTCCCTGACATGGATGTGATTGGAATCTCTGGTAACTTCTGTTCGGACAA  1260

                K  P  A  A  V  N  W  I  E  G  R  G  K  S  V  V  C  E  A  V
1261  GAAACCTGCTGCTGTGAACTGGATTGAGGGACGTGGTAAATCAGTTGTTTGCGAGGCTGT  1320

                I  R  G  E  I  V  N  K  V  L  K  T  S  V  A  A  L  V  E  L
1321  AATCAGAGGAGAGATCGTGAACAAGGTCTTGAAAACGAGCGTGGCTGCTTTAGTCGAGCT  1380

                N  M  L  K  N  L  A  G  S  A  V  A  G  S  L  G  G  F  N  A
1381  CAACATGCTCAAGAACCTAGCTGGCTCTGCTGTTGCAGGCTCTCTAGGTGGATTCAACGC  1440

                H  A  S  N  I  V  S  A  V  F  I  A  T  G  Q  D  P  A  Q  N
1441  TCATGCCAGTAACATAGTGTCTGCTGTATTCATAGCTACTGGCCAAGATCCAGCTCAAAA  1500

                V  E  S  S  Q  C  I  T  M  M  E  A  I  N  D  G  K  D  I  H
1501  CGTGGAGAGTTCTCAATGCATCACCATGATGGAAGCTATTAATGACGGCAAAGATATCCA  1560

                I  S  V  T  M  P  S  I  E  V  G  T  V  G  G  G  T  Q  L  A
1561  TATCTCAGTCACTATGCCATCTATCGAGGTGGGGACAGTGGGAGGAGGAACACAGCTTGC  1620

                S  Q  S  A  C  L  N  L  L  G  V  K  G  A  S  T  E  S  P  G
1621  ATCTCAATCAGCGTGTTTAAACCTGCTCGGAGTTAAAGGAGCAAGCACAGAGTCGCCGGG  1680

                M  N  A  R  R  L  A  T  I  V  A  G  A  V  L  A  G  E  L  S
1681  AATGAACGCAAGGAGGCTAGCGACGATCGTAGCCGGAGCAGTTTTAGCTGGAGAGTTATC  1740

                L  M  S  A  I  A  A  G  Q  L  V  R  S  H  M  K  Y  N  R  S
1741  TTTAATGTCAGCAATTGCAGCTGGACAGCTTGTGAGAAGTCACATGAAATACAATAGATC  1800

                S  R  D  I  S  G  A  T  T  T  T  T  T  T  *
1801  CAGCCGAGACATCTCTGGAGCAACGACAACGACAACAACAACAACATGATCTGAATCTGA  1860

1861  ATCATCATCCTCTCAAAGAAGGACAACAATCCAAAACAAGGGCAGGCTTTTTACAACGCA  1920

1921  TTCACTCAAAACTCGCTGGTGGACAGATTTTAGCCATGTGCGTATGCGTTTGCCCTTTTG  1980

1981  TTAAATAAAAAAACTATTTGTTTTGTTTGTTTGACTTGATATCTTTTTTTGGGATTGAGG  2040

2041  ATTGAGAGAGATAGAGAGATTTTACAAACTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCA  2100

2101  TGGATAATTCGTGTCTCTTTGATTTGTCTAAGGTTTGTCTTTGTTTGTTAGGAAGTGGTC  2160

2161  TATATGAACGAAAAATTTGTGTATGGTGCAGTTGCGTTTGGGGACATTTTTGAGATTTTT  2220

2221  TCTCTGTTTTGTTTCCTCTCTTCGTTTTTATTGTTTGTTACATATAAAATATTTCTCTGT  2280

2281  ATGTTGGAACATCTCTCTCTCTTTAGTTGTTGTTGGTAAAAGATACGGATCTTCTTTCCT  2340

2341  CCAGAAGAATCCATCTATATAATATTACCATCTATGTGTTCTACT                  2385
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FIG. 3CONT'D

SUBSTITUTE SHEET (RULE 26)

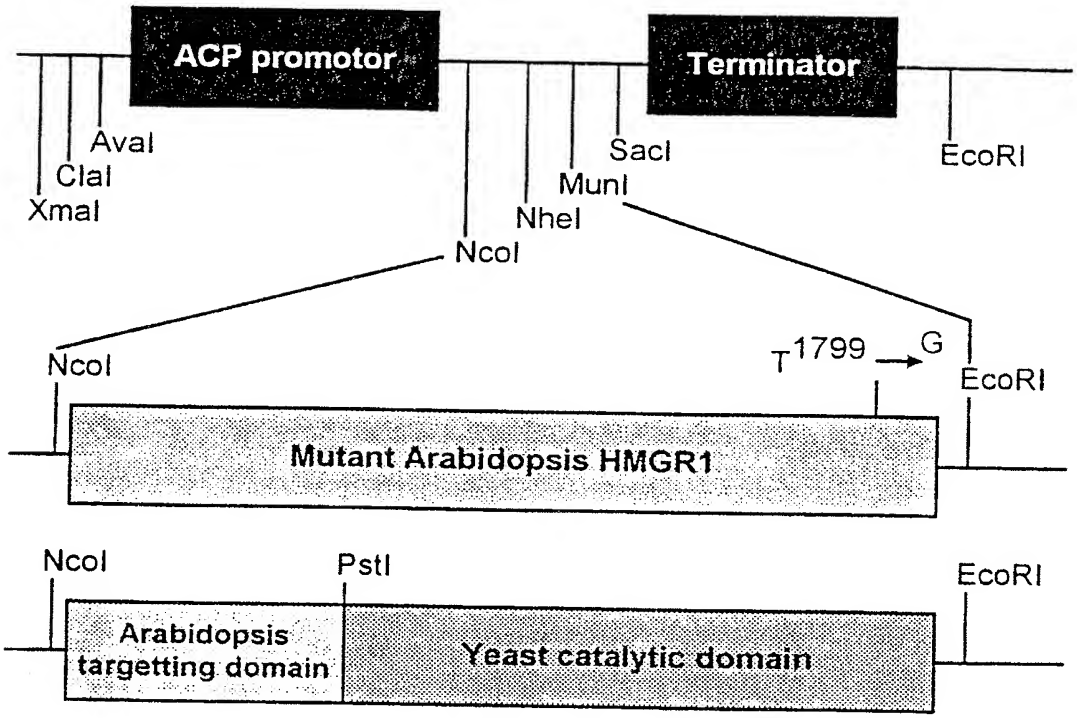

FIG. 4a

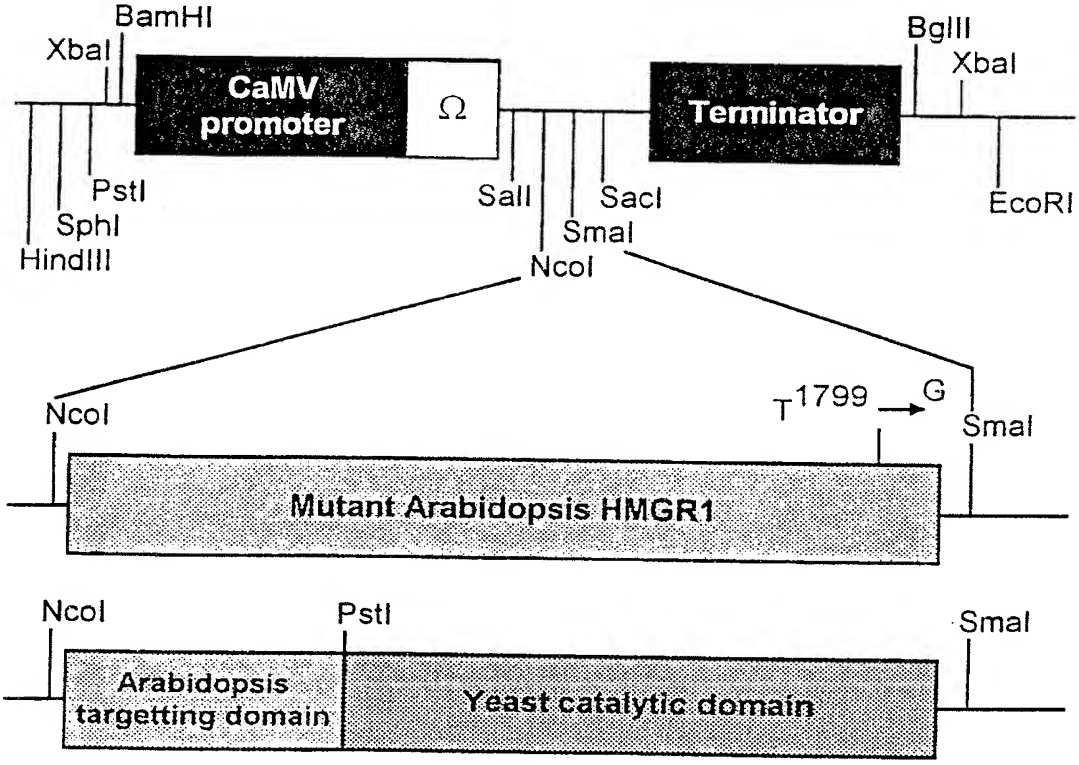

FIG. 4b

FIG. 5a

Nucleotide and derived amino acid sequence of a novel, mutant gene produced by site-directed mutagenesis of Arabidopsis HMGR1

```
      M  D  L  R  R  R  P  P  K  P  P  V  T  N  N  N  N  S  N  G
1     CCATGGATCTCCGTCGGAGGCCTCCTAAACCACCGGTTACCAACAACAACAACTCCAACG     60

      S  F  R  S  Y  Q  P  R  T  S  D  D  D  H  R  R  R  A  T  T
61    GATCTTTCCGTTCTTATCAGCCTCGCACTTCCGATGACGATCATCGTCGCCGGGCTACAA    120

      I  A  P  P  P  K  A  S  D  A  L  P  L  P  L  Y  L  T  N  A
121   CAATTGCTCCTCCACCGAAAGCATCCGACGCGCTTCCTCTTCCGTTATATCTCACAAACG    180

      V  F  F  T  L  F  F  S  V  A  Y  Y  L  L  H  R  W  R  D  K
181   CCGTTTTCTTCACGCTCTTCTTCTCCGTCGCGTATTACCTCCTCCACCGGTGGCGTGACA    240

      I  R  Y  N  T  P  L  H  V  V  T  I  T  E  L  G  A  I  I  A
241   AGATCCGTTACAATACGCCTCTTCACGTCGTCACTATCACAGAACTCGGCGCCATTATTG    300

      L  I  A  S  F  I  Y  L  L  G  F  F  G  I  D  F  V  Q  S  F
301   CTCTCATCGCTTCGTTTATCTATCTCCTAGGGTTTTTTGGTATTGACTTTGTTCAGTCAT    360

      I  S  R  A  S  G  D  A  W  D  L  A  D  T  I  D  D  D  D  H
361   TTATCTCACGTGCCTCTGGTGATGCTTGGGATCTCGCCGATACGATCGATGATGATGACC    420

      R  L  V  T  C  S  P  P  T  P  I  V  S  V  A  K  L  P  N  P
421   ACCGCCTTGTCACGTGCTCTCCACCGACTCCGATCGTTTCCGTTGCTAAATTACCTAATC    480

      E  P  I  V  T  E  S  L  P  E  E  D  E  E  I  V  K  S  V  I
481   CGGAACCTATTGTTACCGAATCGCTTCCTGAGGAAGACGAGGAGATTGTGAAATCGGTTA    540

      D  G  V  I  P  S  Y  S  L  E  S  R  L  G  D  C  K  R  A  A
541   TCGACGGAGTTATTCCATCGTACTCGCTTGAATCTCGTCTCGGTGATTGCAAAAGAGCGG    600

      S  I  R  R  E  A  L  Q  R  V  T  G  R  S  I  E  G  L  P  L
601   CGTCGATTCGTCGTGAGGCGTTGCAGAGAGTCACCGGGAGATCGATTGAAGGGTTACCGT    660

      D  G  F  D  Y  E  S  I  L  G  Q  C  C  E  M  P  V  G  Y  I
661   TGGATGGATTTGATTATGAATCGATTTTGGGGCAATGCTGTGAGATGCCTGTTGGATACA    720

      Q  I  P  V  G  I  A  G  P  L  L  L  D  G  Y  E  Y  S  V  P
721   TTCAGATTCCTGTTGGGATTGCTGGTCCATTGTTGCTTGATGGTTATGAGTACTCTGTTC    780

      M  A  T  T  E  G  C  L  V  A  S  T  N  R  G  C  K  A  M  F
781   CTATGGCTACAACCGAAGGTTGTTTGGTTGCTAGCACTAACAGAGGCTGCAAGGCTATGT    840
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```
          I  S  G  G  A  T  S  T  V  L  K  D  G  M  T  R  A  P  V  V
841  TTATCTCTGGTGGCGCCACCAGTACCGTTCTTAAGGACGGTATGACCCGAGCACCTGTTG  900

          R  F  A  S  A  R  R  A  S  E  L  K  F  F  L  E  N  P  E  N
901  TTCGGTTCGCTTCGGCGAGACGAGCTTCGGAGCTTAAGTTTTTCTTGGAGAATCCAGAGA  960

          F  D  T  L  A  V  V  F  N  R  S  S  R  F  A  R  L  Q  S  V
961  ACTTTGATACTTTGGCAGTAGTCTTCAACAGGTCGAGTAGATTTGCAAGACTGCAAAGTG  1020

          K  C  T  I  A  G  K  N  A  Y  V  R  F  C  C  S  T  G  D  A
1021 TTAAATGCACAATCGCGGGGAAGAATGCTTATGTAAGGTTCTGTTGTAGTACTGGTGATG  1080

          M  G  M  N  M  V  S  K  G  V  Q  N  V  L  E  Y  L  T  D  D
1081 CTATGGGGATGAATATGGTTTCTAAAGGTGTGCAGAATGTTCTTGAGTATCTTACCGATG  1140

          F  P  D  M  D  V  I  G  I  S  G  N  F  C  S  D  K  K  P  A
1141 ATTTCCCTGACATGGATGTGATTGGAATCTCTGGTAACTTCTGTTCGGACAAGAAACCTG  1200

          A  V  N  W  I  E  G  R  G  K  S  V  V  C  E  A  V  I  R  G
1201 CTGCTGTGAACTGGATTGAGGGACGTGGTAAATCAGTTGTTTGCGAGGCTGTAATCAGAG  1260

          E  I  V  N  K  V  L  K  T  S  V  A  A  L  V  E  L  N  M  L
1261 GAGAGATCGTGAACAAGGTCTTGAAAACGAGCGTGGCTGCTTTAGTCGAGCTCAACATGC  1320

          K  N  L  A  G  S  A  V  A  G  S  L  G  G  F  N  A  H  A  S
1321 TCAAGAACCTAGCTGGCTCTGCTGTTGCAGGCTCTCTAGGTGGATTCAACGCTCATGCCA  1380

          N  I  V  S  A  V  F  I  A  T  G  Q  D  P  A  Q  N  V  E  S
1381 GTAACATAGTGTCTGCTGTATTCATAGCTACTGGCCAAGATCCAGCTCAAAACGTGGAGA  1440

          S  Q  C  I  T  M  M  E  A  I  N  D  G  K  D  I  H  I  S  V
1441 GTTCTCAATGCATCACCATGATGGAAGCTATTAATGACGGCAAAGATATCCATATCTCAG  1500

          T  M  P  S  I  E  V  G  T  V  G  G  G  T  Q  L  A  S  Q  S
1501 TCACTATGCCATCTATCGAGGTGGGGACAGTGGGAGGAGGAACACAGCTTGCATCTCAAT  1560

          A  C  L  N  L  L  G  V  K  G  A  S  T  E  S  P  G  M  N  A
1561 CAGCGTGTTTAAACCTGCTCGGAGTTAAAGGAGCAAGCACAGAGTCGCCGGGAATGAACG  1620

          R  R  L  A  T  I  V  A  G  A  V  L  A  G  E  L  S  L  M  S
1621 CAAGGAGGCTAGCGACGATCGTAGCCGGAGCAGTTTTAGCTGGAGAGTTATCTTTAATGT  1680

          A  I  A  A  G  Q  L  V  R  S  H  M  K  Y  N  R  A  S  R  D
1681 CAGCAATTGCAGCTGGACAGCTTGTGAGAAGTCACATGAAATACAATAGAGCCAGCCGAG  1740

          I  S  G  A  T  T  T  T  T  T  T  T  *
1741 ACATCTCTGGAGCAACGACAACGACAACAACAACAACATGATCTGAATCTGAATTC  1796
```

FIG. 5a CONT'D

FIG. 5b

Nucleotide and derived amino acid sequence of a novel sequence comprising part of *Arabidopsis thaliana* HMGR1 and part of yeast HMGR1

```
      M  D  L  R  R  R  P  P  K  P  P  V  T  N  N  N  N  S  N  G
1     CCATGGATCTCCGTCGGAGGCCTCCTAAACCACCGGTTACCAACAACAACAACTCCAACG    60

      S  F  R  S  Y  Q  P  R  T  S  D  D  D  H  R  R  R  A  T  T
61    GATCTTTCCGTTCTTATCAGCCTCGCACTTCCGATGACGATCATCGTCGCCGGGCTACAA   120

      I  A  P  P  P  K  A  S  D  A  L  P  L  P  L  Y  L  T  N  A
121   CAATTGCTCCTCCACCGAAAGCATCCGACGCGCTTCCTCTTCCGTTATATCTCACAAACG   180

      V  F  F  T  L  F  F  S  V  A  Y  Y  L  L  H  R  W  R  D  K
181   CCGTTTTCTTCACGCTCTTCTTCTCCGTCGCGTATTACCTCCTCCACCGGTGGCGTGACA   240

      I  R  Y  N  T  P  L  H  V  V  T  I  T  E  L  G  A  I  I  A
241   AGATCCGTTACAATACGCCTCTTCACGTCGTCACTATCACAGAACTCGGCGCCATTATTG   300

      L  I  A  S  F  I  Y  L  L  G  F  F  G  I  D  F  V  Q  S  F
301   CTCTCATCGCTTCGTTTATCTATCTCCTAGGGTTTTTTGGTATTGACTTTGTTCAGTCAT   360

      I  S  R  A  S  G  D  A  W  D  L  A  D  T  I  D  D  D  D  H
361   TTATCTCACGTGCCTCTGGTGATGCTTGGGATCTCGCCGATACGATCGATGATGATGACC   420

      R  L  V  T  C  S  P  P  T  P  I  V  S  V  A  K  L  P  N  P
421   ACCGCCTTGTCACGTGCTCTCCACCGACTCCGATCGTTTCCGTTGCTAAATTACCTAATC   480

                  Arabidopsis ←      → Yeast
      E  P  I  V  T  E  S  L  P  A  E  N  K  E  V  A  A  L  V  I
481   CGGAACCTATTGTTACCGAATCGCTTCCTGCAGAGAACAAAGAGGTCGCTGCCTTGGTTA   540

      H  G  K  L  P  L  Y  A  L  E  K  K  L  G  D  T  T  R  A  V
541   TTCACGGTAAGTTACCTTTGTACGCTTTGGAGAAAAAATTAGGTGATACTACGAGAGCGG   600

      A  V  R  R  K  A  L  S  I  L  A  E  A  P  V  L  A  S  D  R
601   TTGCGGTACGTAGGAAGGCTCTTTCAATTTTGGCAGAAGCTCCTGTATTAGCATCTGATC   660

      L  P  Y  K  N  Y  D  Y  D  R  V  F  G  A  C  C  E  N  V  I
661   GTTTACCATATAAAAATTATGACTACGACCGCGTATTTGGCGCTTGTTGTGAAAATGTTA   720
```

```
          G  Y  M  P  L  P  V  G  V  I  G  P  L  V  I  D  G  T  S  Y
721  TAGGTTACATGCCTTTGCCCGTTGGTGTTATAGGCCCCTTGGTTATCGATGGTACATCTT  780

          H  I  P  M  A  T  T  E  G  C  L  V  A  S  A  M  R  G  C  K
781  ATCATATACCAATGGCAACTACAGAGGGTTGTTTGGTAGCTTCTGCCATGCGTGGCTGTA  840

          A  I  N  A  G  G  G  A  T  T  V  L  T  K  D  G  M  T  R  G
841  AGGCAATCAATGCTGGCGGTGGTGCAACAACTGTTTTAACTAAGGATGGTATGACAAGAG  900

          P  V  V  R  F  P  T  L  K  R  S  G  A  C  K  I  W  L  D  S
901  GCCCAGTAGTCCGTTTCCCAACTTTGAAAAGATCTGGTGCCTGTAAGATATGGTTAGACT  960

          E  E  G  Q  N  A  I  K  K  A  F  N  S  T  S  R  F  A  R  L
961  CAGAAGAGGGACAAAACGCAATTAAAAAAGCTTTTAACTCTACATCAAGATTTGCACGTC  1020

          Q  H  I  Q  T  C  L  A  G  D  L  L  F  M  R  F  R  T  T  T
1021 TGCAACATATTCAAACTTGTCTAGCAGGAGATTTACTCTTCATGAGATTTAGAACAACTA  1080

          G  D  A  M  G  M  N  M  I  S  K  G  V  E  Y  S  L  K  Q  M
1081 CTGGTGACGCAATGGGTATGAATATGATTTCTAAAGGTGTCGAATACTCATTAAAGCAAA  1140

          V  E  E  Y  G  W  E  D  M  E  V  V  S  V  S  G  N  Y  C  T
1141 TGGTAGAAGAGTATGGCTGGGAAGATATGGAGGTTGTCTCCGTTTCTGGTAACTACTGTA  1200

          D  K  K  P  A  A  I  N  W  I  E  G  R  G  K  S  V  V  A  E
1201 CCGACAAAAAACCAGCTGCCATCAACTGGATCGAAGGTCGTGGTAAGAGTGTCGTCGCAG  1260

          A  T  I  P  G  D  V  V  R  K  V  L  K  S  D  V  S  A  L  V
1261 AAGCTACTATTCCTGGTGATGTTGTCAGAAAAGTGTTAAAAAGTGATGTTTCCGCATTGG  1320

          E  L  N  I  A  K  N  L  V  G  S  A  M  A  G  S  V  G  G  F
1321 TTGAGTTGAACATTGCTAAGAATTTGGTTGGATCTGCAATGGCTGGGTCTGTTGGTGGAT  1380

          N  A  H  A  A  N  L  V  T  A  V  F  L  A  L  G  Q  D  P  A
1381 TTAACGCACATGCAGCTAATTTAGTGACAGCTGTTTTCTTGGCATTAGGACAAGATCCTG  1440

          Q  N  V  E  S  S  N  C  I  T  L  M  K  E  V  D  G  D  L  R
1441 CACAAAATGTTGAAAGTTCCAACTGTATAACATTGATGAAAGAAGTGGACGGTGATTTGA  1500

          I  S  V  S  M  P  S  I  E  V  G  T  I  G  G  G  T  V  L  E
1501 GAATTTCCGTATCCATGCCATCCATCGAAGTAGGTACCATCGGTGGTGGTACTGTTCTAG  1560

          P  Q  G  A  M  L  D  L  L  G  V  R  G  P  H  A  T  A  P  G
1561 AACCACAAGGTGCCATGTTGGACTTATTAGGTGTAAGAGGCCCGCATGCTACCGCTCCTG  1620

          T  N  A  R  Q  L  A  R  I  V  A  C  A  V  L  A  G  E  L  S
1621 GTACCAACGCACGTCAATTAGCAAGAATAGTTGCCTGTGCCGTCTTGGCAGGTGAATTAT  1680

          L  C  A  A  L  A  A  G  H  L  V  Q  S  H  M  T  H  N  R  K
1681 CCTTATGTGCTGCCCTAGCAGCCGGCCATTTGGTTCAAAGTCATATGACCCACAACAGGA  1740

          P  A  E  P  T  K  P  N  N  L  D  A  T  D  I  N  R  L  K  D
1741 AACCTGCTGAACCAACAAAACCTAACAATTTGGACGCCACTGATATAAATCGTTTGAAAG  1800

          G  S  V  T  C  I  K  S  *
1801 ATGGGTCCGTCACCTGCATTAAATCCTAAACTTAGTCATACGTCGAATTC  1850
```

FIG. 5b CONT'D

FIG. 6

```
athmgr1 .......... .......... .......... ........MD  2
athmgrm .......... .......... .......... ........MD  2
chimaeric .......... .......... .......... ........MD  2
schmgr1 NLSVVVIIMK LSVILLFVFI NFYNFGANWV NDAFNSLYFG 439

athmgr1 LRRRPPKPPV TNNNNSN... .......... ..GSFRSYQP  27
athmgrm LRRRPPKPPV TNNNNSN... .......... ..GSFRSYQP  27
chimaeric LRRRPPKPPV TNNNNSN... .......... ..GSFRSYQP  27
schmgr1 KERVSLPDFI TSNASENFKE QAIVSVTPLL YYKPIKSYQR 479

athmgr1 RTSDDDHRRR ATTIAPPPKA SDALPLPLYL TNAVFFTLFF  67
athmgrm RTSDDDHRRR ATTIAPPPKA SDALPLPLYL TNAVFFTLFF  67
chimaeric RTSDDDHRRR ATTIAPPPKA SDALPLPLYL TNAVFFTLFF  67
schmgr1 IEDMVLLLLR NVSVAIRDRF VSKLVLSALV CSAV...... 513

athmgr1 SVAYYLLHRW RDKIRYNTPL HV...VTITE LGAIIALIAS 104
athmgrm SVAYYLLHRW RDKIRYNTPL HV...VTITE LGAIIALIAS 104
chimaeric SVAYYLLHRW RDKIRYNTPL HV...VTITE LGAIIALIAS 104
schmgr1 .INVYLLNAA RIHTSYTADQ LVKTEVTKKS FTAPVQKAST 552

athmgr1 FIYLLGFFGI DFVQSFISRA SGDAWDLADT IDDDDHRLVT 144
athmgrm FIYLLGFFGI DFVQSFISRA SGDAWDLADT IDDDDHRLVT 144
chimaeric FIYLLGFFGI DFVQSFISRA SGDAWDLADT IDDDDHRLVT 144
schmgr1 PVLTNKTVIS GSKVKSLSSA QSSSSGPSSS SEEDDSRDIE 592

athmgr1 CSPPTPIVSV AKLPNPEPIV TESLPEE..D EEIVKSVIDG 182
athmgrm CSPPTPIVSV AKLPNPEPIV TESLPEE..D EEIVKSVIDG 182
chimaeric CSPPTPIVSV AKLPNPEPIV TESLPAE..N KEVAALVIHG 182
schmgr1 SLDK....KI RPLEELEALL SSGNTKQLKN KEVAALVIHG 628

athmgr1 VIPSYSLESR LGDCKRAASI RREALQRVTG RSI...EGLP 219
athmgrm VIPSYSLESR LGDCKRAASI RREALQRVTG RSI...EGLP 219
chimaeric KLPLYALEKK LGDTTRAVAV RRKALSILAE APVLASDRLP 222
schmgr1 KLPLYALEKK LGDTTRAVAV RRKALSILAE APVLASDRLP 668

athmgr1 LDGFDYESIL GQCCEMPVGY IQIPVGIAGP LLLDGYEYSV 259
athmgrm LDGFDYESIL GQCCEMPVGY IQIPVGIAGP LLLDGYEYSV 259
chimaeric YKNYDYDRVF GACCENVIGY MPLPVGVIGP LVIDGTSYHI 262
schmgr1 YKNYDYDRVF GACCENVIGY MPLPVGVIGP LVIDGTSYHI 708
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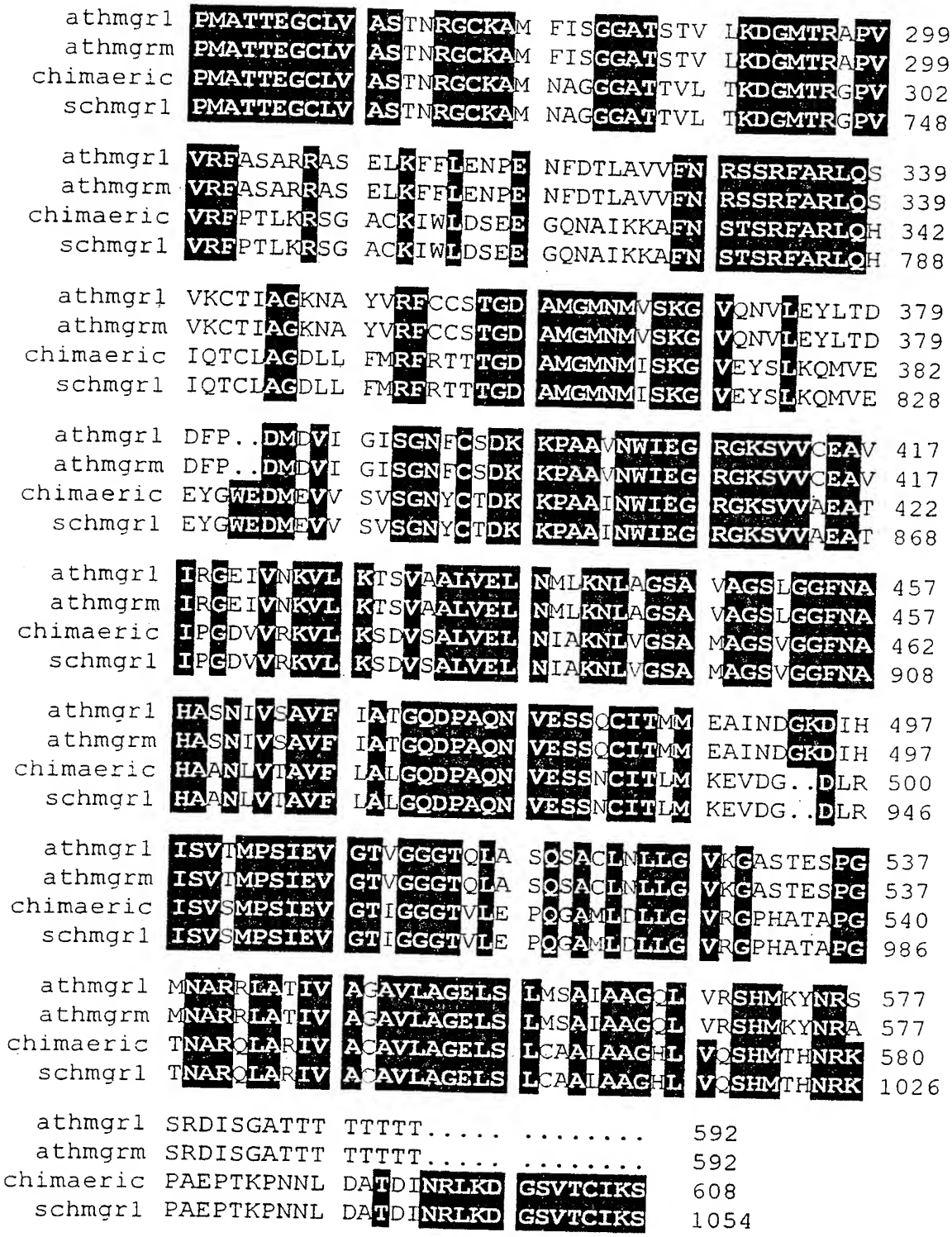


FIG. 6CONT'D

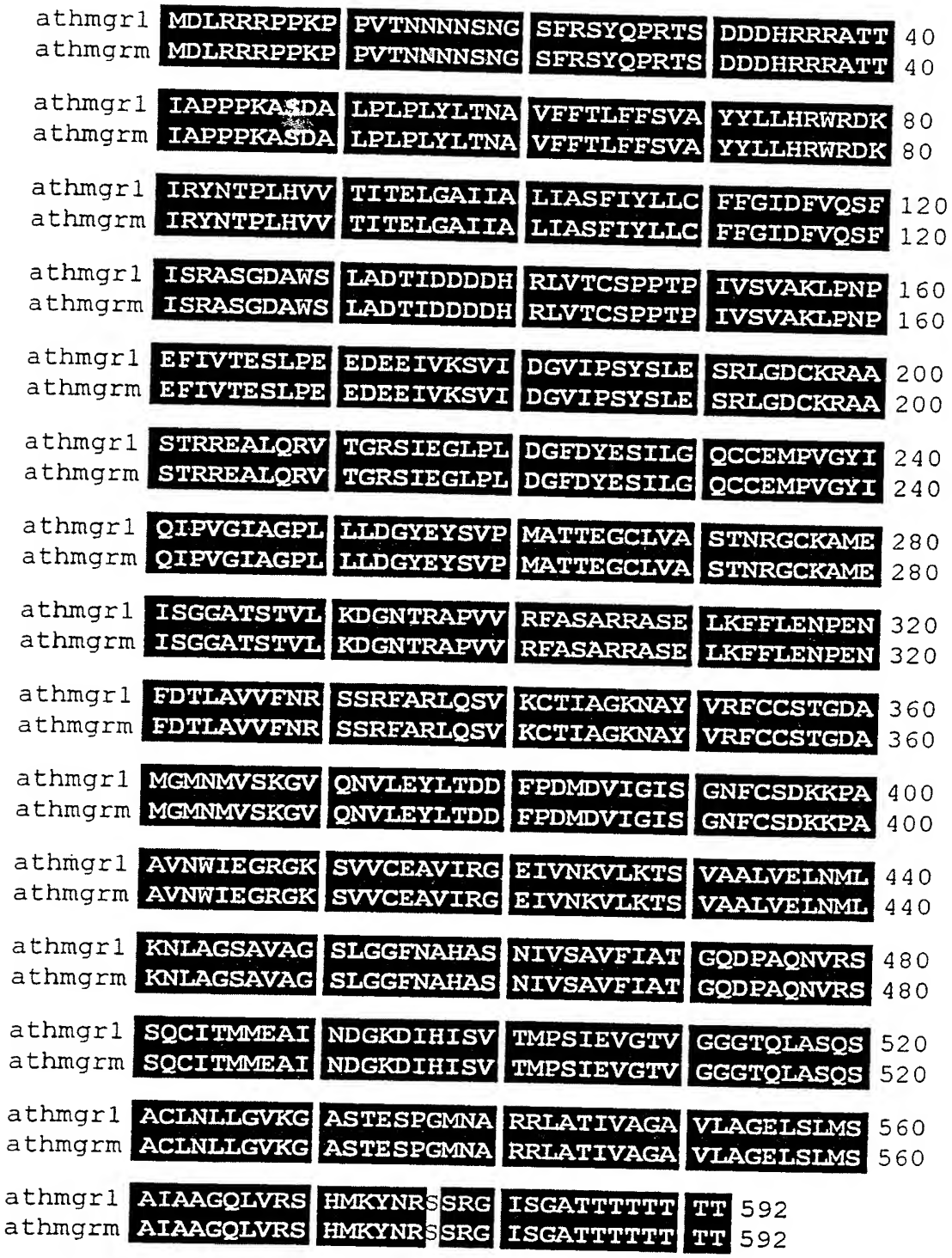
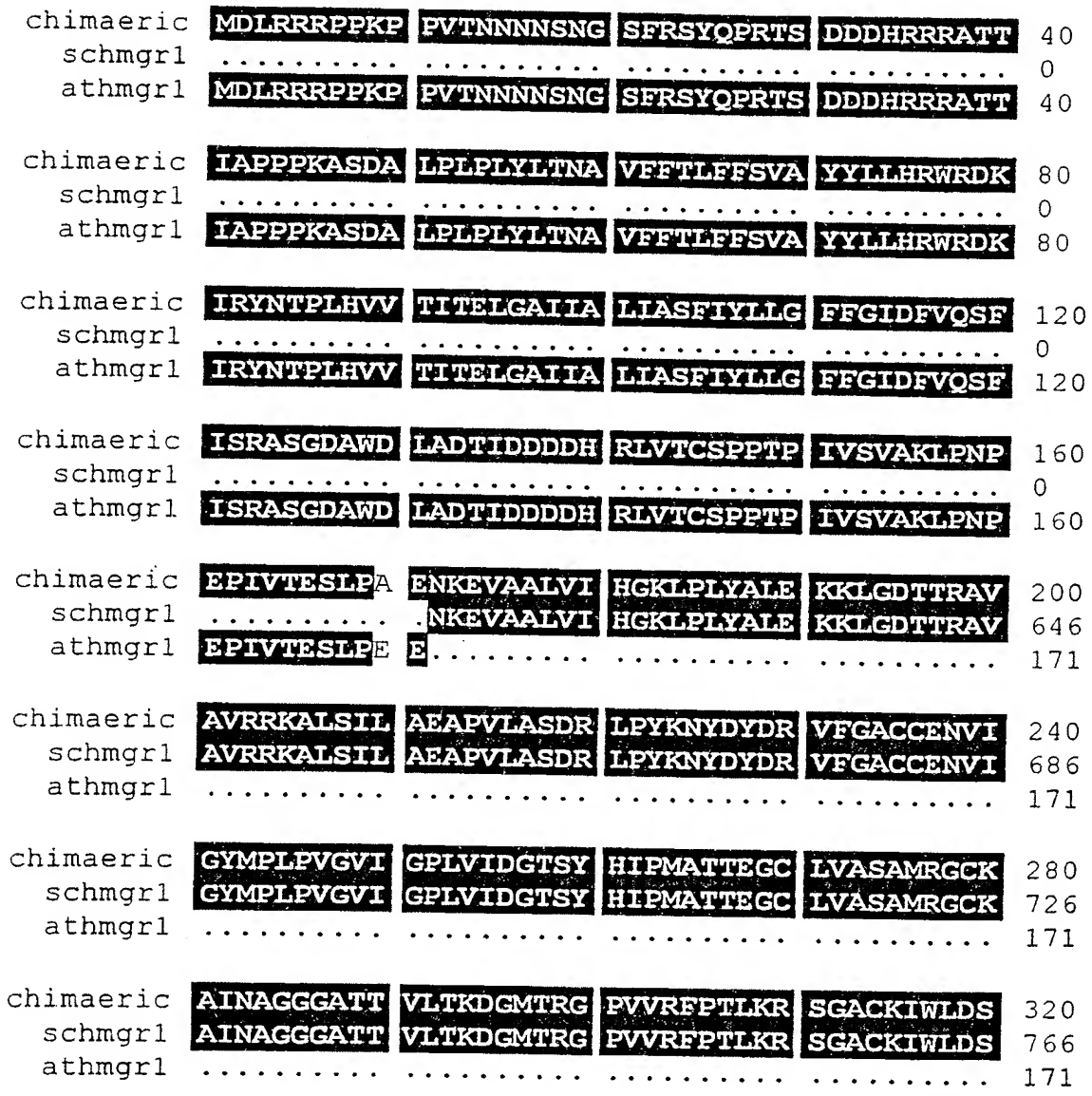


FIG. 7

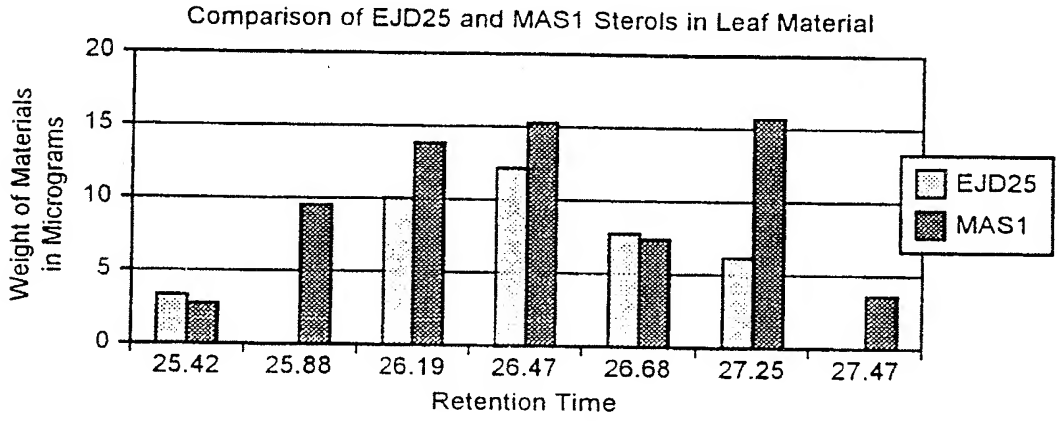
FIG. 8



```
chimaeric EEGQNAIKKA FNSTSRFARL QHIQTCLAGD LLFMRFRTTT 360
  schmgr1 EEGQNAIKKA FNSTSRFARL QHIQTCLAGD LLFMRFRTTT 806
  athmgr1 .......... .......... .......... .......... 171

chimaeric GDAMGMNMIS KGVEYSLKQM VEEYGWEDME VVSVSGNYCT 400
  schmgr1 GDAMGMNMIS KGVEYSLKQM VEEYGWEDME VVSVSGNYCT 846
  athmgr1 .......... .......... .......... .......... 171

chimaeric DKKPAAINWI EGRGKSVVAE ATIPGDVVRK VLKSDVSALV 440
  schmgr1 DKKPAAINWI EGRGKSVVAE ATIPGDVVRK VLKSDVSALV 886
  athmgr1 .......... .......... .......... .......... 171

chimaeric ELNIAKNLVG SAMAGSVGGF NAHAANLVTA VFLALGQDPA 480
  schmgr1 ELNIAKNLVG SAMAGSVGGF NAHAANLVTA VFLALGQDPA 926
  athmgr1 .......... .......... .......... .......... 171

chimaeric QNVESSNCIT LMKEVDGDLR ISVSMPSIEV GTIGGGTVLE 520
  schmgr1 QNVESSNCIT LMKEVDGDLR ISVSMPSIEV GTIGGGTVLE 966
  athmgr1 .......... .......... .......... .......... 171

chimaeric PQGAMLDLLG VRGPHATAPG TNARQLARIV ACAVLAGELS 560
  schmgr1 PQGAMLDLLG VRGPHATAPG TNARQLARIV ACAVLAGELS 966
  athmgr1 .......... .......... .......... .......... 171

chimaeric LCAALAAGHI VQSHMTNRK PAEPTKPNNL DATDINRLKD 600
  schmgr1 LCAALAAGHI VQSHMTNRK PAEPTKPNNL DATDINRLKD 429
  athmgr1 .......... .......... .......... .......... 171

chimaeric GSVTCIKS 608
  schmgr1 GSVTCIKS 1046
  athmgr1 ........ 171
```

FIG. 8CONT'D

Assignment	Retention Time		Weight			% of dry sample weight	
campesterol	25.42		3.3	2.8		0.0066	0.0056
Unknown	25.88		0	9.5		0	0.019
stigmasterol	26.19		10	13.9		0.02	0.0278
beta-sitosterol	26.47		12.1	15.3		0.0242	0.0306
iso-fucosterol	26.68		7.76	7.4		0.0156	0.0148
Hydrocarbon	27.25		6.2	15.7		0.0124	0.0314
Hydrocarbon	27.47		0	3.6		0	0.0072
			EJD25	MAS1		EJD25	MAS1



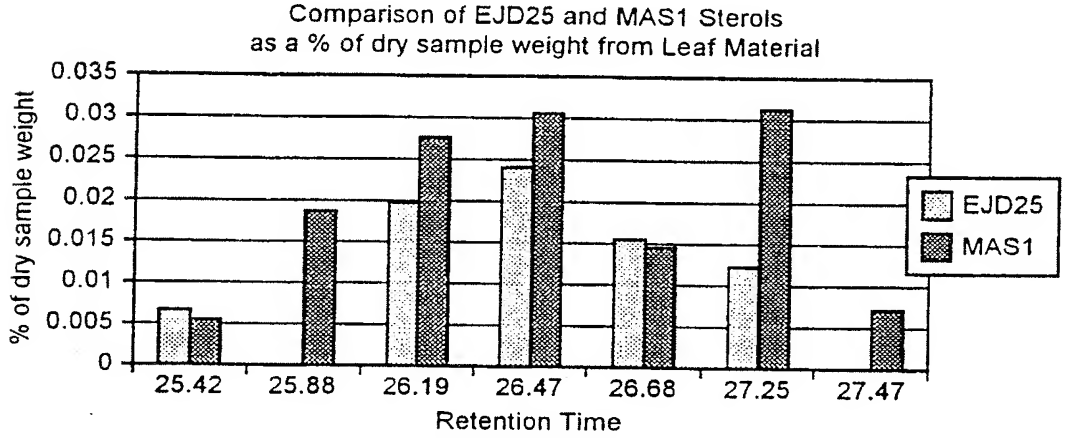
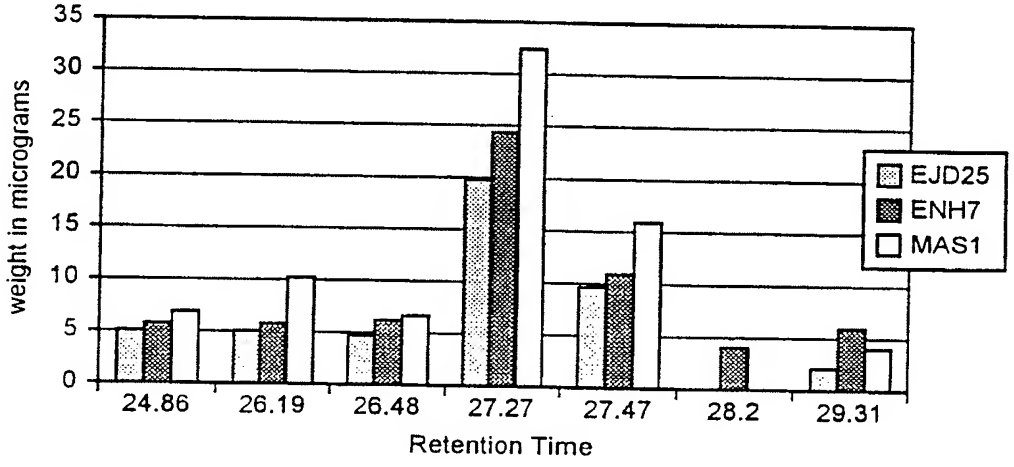


FIG. 9

Assignment	Retention Time		Weight			% of dry sample weight		
standard	25.19	500	500	500				
cholesterol	24.86	5.06	5.8	6.8		0.0096	0.011	0.013
campesterol	26.19	5.09	5.86	10.33		0.01	0.011	0.02
stigmasterol	26.48	4.68	6.21	6.63		0.00914	0.012	0.013
beta-sitosterol	27.27	19.86	24.46	32.6		0.0387	0.048	0.063
iso-fucosterol	27.47	9.8	10.94	16.12		0.019	0.021	0.031
cycloartenol	28.2	0	4.12			0	0.0079	
24-ethylidene lophenol	29.31	1.92	6.01	3.92		0.00368	0.012	0.0075
		EJD25	ENH7	MAS1		EJD25	ENH7	MAS1



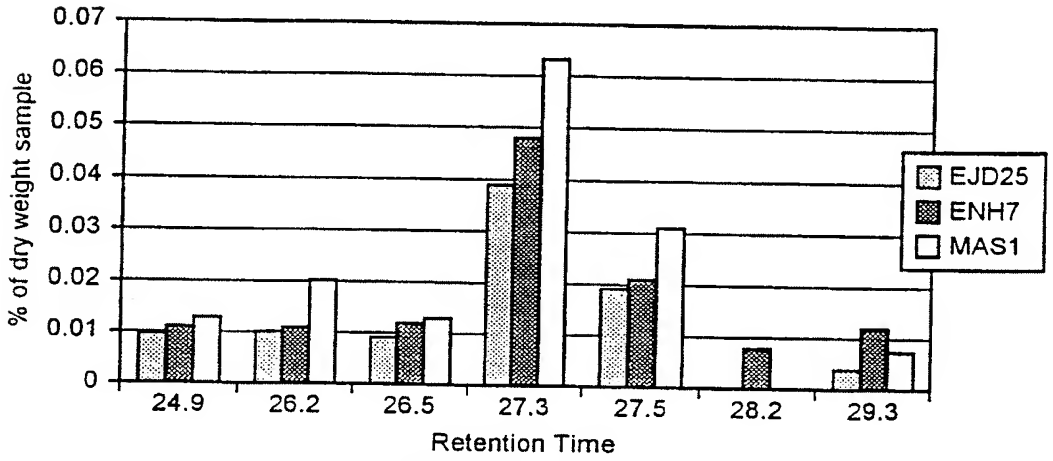


FIG. 10

INTERNATIONAL SEARCH REPORT

Inter. ɔnal Application No
PCT/GB 00/04141

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/53 C12N9/04 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHAPPELL JOSEPH ET AL: "Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants?" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 109, no. 4, 1995, pages 1337-1343, XP002133625 ISSN: 0032-0889 the whole document ---	1,4,5, 8-10, 17-26, 28-30
X	US 5 589 619 A (WOLF FRED R ET AL) 31 December 1996 (1996-12-31)	1,4,5, 8-10, 17-26, 28-31, 39,40, 43,44
Y	the whole document ---	41,42

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 2001	26/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Inter. ›nal Application No
PCT/GB 00/04141

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 98 45457 A (MONSANTO CO) 15 October 1998 (1998-10-15) page 17 -page 21; tables 5,6	29 41,42
X	SCHALLER HUBERT ET AL: "Expression of the Hevea brasiliensis (H.B.K.) Mull. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 109, no. 3, 1995, pages 761-770, XP002133624 ISSN: 0032-0889 the whole document	1,8-10, 17-26, 28-30
X	OMKUMAR RAMAKRISHNAPILLAI V ET AL: "Modulation of Syrian hamster 3-hydroxy-3-methylglutaryl-CoA reductase activity by phosphorylation: Role of serine 871." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 9, 1994, pages 6810-6814, XP002159997 ISSN: 0021-9258 the whole document	32,37
X	SATO RYUICHIRO ET AL: "Replacement of serine-871 of hamster 3-hydroxy-3-methylglutaryl-CoA reductase prevents phosphorylation by AMP-activated kinase and blocks inhibition of sterol synthesis induced by ATP depletion." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 90, no. 20, 1993, pages 9261-9265, XP002159998 1993 ISSN: 0027-8424 the whole document	32,37
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1979 VU C V ET AL: "EFFECTS OF INHIBITORS ON THE BIOSYNTHESIS OF STEROLS REDUCING SUGARS AND CHLOROPHYLL AND THE DEVELOPMENT OF ISO CITRATE LYASE IN GERMINATING SEEDS OF LONGLEAF PINE PINUS-PALUSTRIS" Database accession no. PREV198070005363 XP002145635 abstract & PLANT SCIENCE LETTERS, vol. 16, no. 2-3, 1979, pages 255-266, ISSN: 0304-4211	30

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6

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/GB 00/04141

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ABIDI S L ET AL: "Effect of genetic modification on the distribution of minor constituents in canola oil." JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 76, no. 4, April 1999 (1999-04), pages 463-467, XP002159999 ISSN: 0003-021X the whole document	29-31
P,X	WO 00 61771 A (MONSANTO CO) 19 October 2000 (2000-10-19) page 98 -page 105	1,8-10, 17-26, 28-31
A	DATABASE WPI Section Ch, Week 199729 Derwent Publications Ltd., London, GB; Class C06, AN 1997-314223 XP002160017 & JP 09 121863 A (SUMITOMO CHEM CO LTD), 13 May 1997 (1997-05-13) abstract	1-38
A	SUGDEN CHRISTOPHER ET AL: "Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro." PLANT PHYSIOLOGY (ROCKVILLE), vol. 120, no. 1, May 1999 (1999-05), pages 257-274, XP002160000 ISSN: 0032-0889 the whole document	1-38
A	HALFORD NIGEL F ET AL: "SNF1-related protein kinases: Global regulators of carbon metabolism in plants?" PLANT MOLECULAR BIOLOGY, vol. 37, no. 5, July 1998 (1998-07), pages 735-748, XP000982082 ISSN: 0167-4412 the whole document	1-38
A	RE EDWARD B ET AL: "Co-expression of native and introduced genes reveals cryptic regulation of HMG CoA reductase expression in Arabidopsis" PLANT JOURNAL,GB,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 7, no. 5, 1995, pages 771-784, XP002133632 ISSN: 0960-7412 the whole document	1-38

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/GB 00/04141

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; October 1998 (1998-10) GODOY-HERNANDEZ GREGORIO C ET AL: "Antisense expression of hmg1 from Arabidopsis thaliana encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, reduces isoprenoid production in transgenic tobacco plants." Database accession no. PREV199900035851 XP002158648 abstract & JOURNAL OF PLANT PHYSIOLOGY, vol. 153, no. 3-4, October 1998 (1998-10), pages 415-424, ISSN: 0176-1617	1-38

A	SCHALLER H ET AL: "Overexpression of an Arabidopsis cDNA encoding a sterol-C24(1)-methyltransferase in tobacco modifies the ratio of 24-methyl cholesterol to sitosterol and is associated with growth reduction" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 118, no. 2, October 1998 (1998-10), pages 461-469, XP002130682 ISSN: 0032-0889 the whole document	1-38

A	GONDET LAURENCE ET AL: "Regulation of sterol content in membranes by subcellular compartmentation of steryl-esters accumulating in a sterol-overproducing tobacco mutant" PLANT PHYSIOLOGY,ROCKWILLE, MD,US, vol. 105, no. 2, 1994, pages 509-518, XP002133633 ISSN: 0032-0889 the whole document	1-38

A	OMKUMAR RAMAKRISHNAPILLAI V ET AL: "Phosphorylation of Ser-871 impairs the function of His-865 of Syrian hamster 3-hydroxy-3-methylglutaryl-CoA reductase." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 24, 1994, pages 16862-16866, XP002160003 ISSN: 0021-9258 figure 1	1-38

	-/--	

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/GB 00/04141

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 1998 (1998-01) POLAKOWSKI T ET AL: "Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast." Database accession no. PREV199800141339 XP002133626 abstract & APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 49, no. 1, January 1998 (1998-01), pages 66-71, ISSN: 0175-7598 -----	1-38

6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/GB 00/04141

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5589619 A	31-12-1996	US 5349126 A	20-09-1994
		US 5306862 A	26-04-1994
		AU 653748 B	13-10-1994
		AU 8561991 A	16-04-1992
		CA 2052792 A	13-04-1992
		EP 0480730 A	15-04-1992
		JP 5115298 A	14-05-1993
		MX 9101504 A	01-07-1992
		TR 25647 A	01-07-1993
		US 5365017 A	15-11-1994
		ZA 9107925 A	26-08-1992
WO 9845457 A	15-10-1998	AU 724046 B	07-09-2000
		AU 5709998 A	30-10-1998
		BR 9714439 A	21-03-2000
		CN 1247569 A	15-03-2000
		EP 0958370 A	24-11-1999
WO 0061771 A	19-10-2000	NONE	
JP 9121863 A	13-05-1997	NONE	